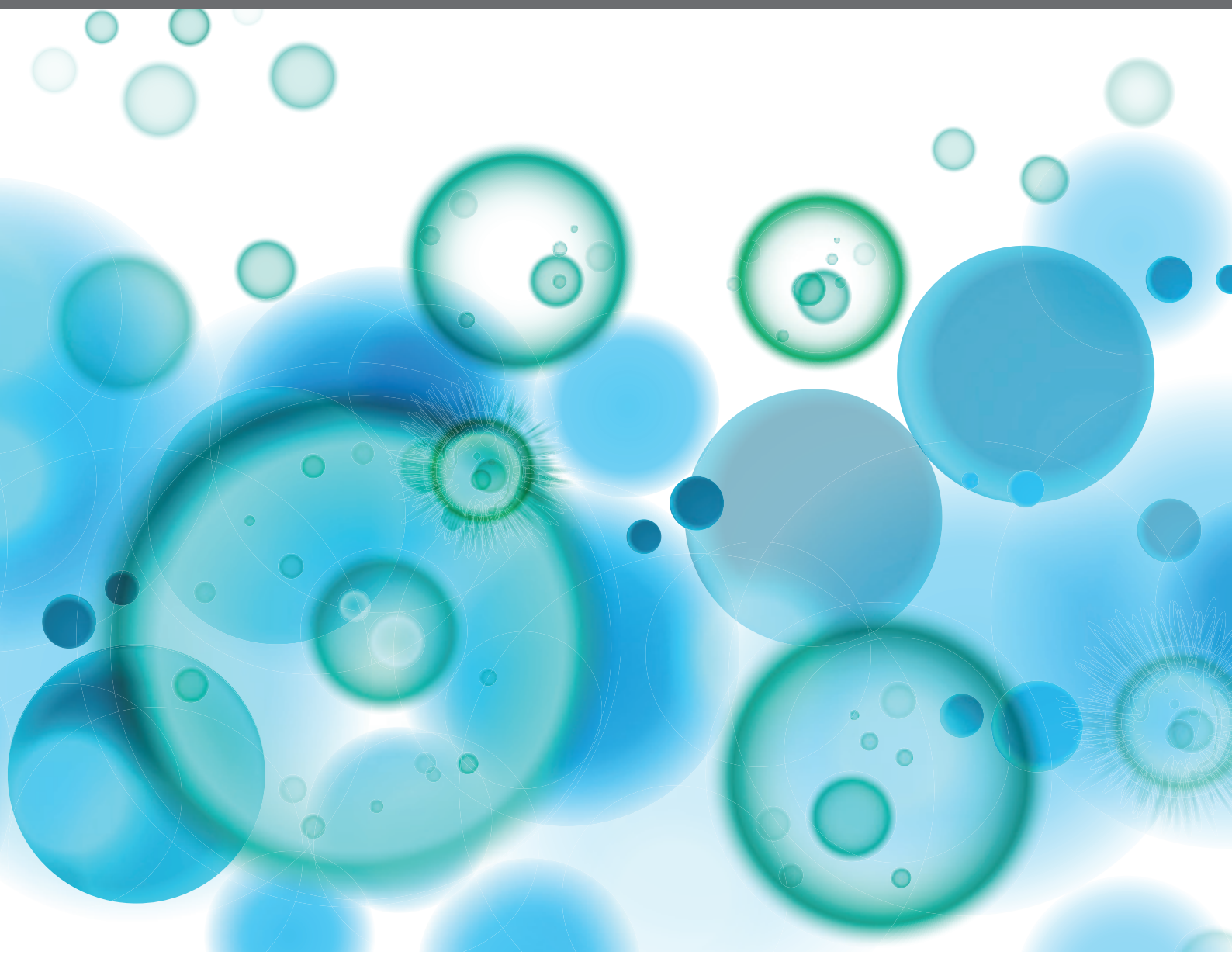
MOLECULAR MECHANISMS OF DENDRITIC CELL-MEDIATED IMMUNE TOLERANCE AND AUTOIMMUNITY, 2nd Edition

EDITED BY: Fang Zhou, Femke Broere and Dipyaman Ganguly

PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-8325-4770-0
DOI 10.3389/978-2-8325-4770-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

MOLECULAR MECHANISMS OF DENDRITIC CELL-MEDIATED IMMUNE TOLERANCE AND AUTOIMMUNITY, 2nd Edition

Topic Editors:
Fang Zhou, CAS Lamvac Biotech Co., Ltd., China
Femke Broere, Utrecht University, Netherlands
Dipyaman Ganguly, Indian Institute of Chemical Biology (CSIR), India

Topic Editor Dr. Zhou is Employed by Cas Lamvac Biotech Co. Ltd. The Other Topic Editors Declare No Conflict of Interest With Regard to the Research Topic Theme.

Publisher's note: This is a 2nd edition due to an article retraction.

Citation: Zhou, F., Broere, F., Ganguly, D., eds. (2024). Molecular Mechanisms of Dendritic Cell-Mediated Immune Tolerance and Autoimmunity, 2nd Edition. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4770-0

Table of Contents

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 July 2020
doi: 10.3389/fimmu.2020.01436

NonO Is a Novel Co-factor of PRDM1 and Regulates Inflammatory Response in Monocyte Derived-Dendritic Cells

Kyungwoo Lee[1], *Su Hwa Jang*[1,2], *Hong Tian*[1] *and Sun Jung Kim*[1]*

[1] Institute of Molecular Medicine, The Feinstein Institute for Medical Research, Manhasset, NY, United States, [2] Department of Biomedical Science, Graduate School of Biomedical Sciences and Engineering, Hanyang University, Seoul, South Korea

Proper expression of the transcription factor, Positive regulatory domain 1 (*PRDM1*), is required for maintaining homeostasis of human monocyte derived-dendritic cells (MO-DCs). The molecular mechanisms and gene targets of PRDM1 in B and T lymphocytes have been identified. However, the function of PRDM1 in dendritic cells (DCs) remains unclear. We investigate co-regulators of PRDM1 in MO-DCs identified by mass spectrometry (MS) and co-immunoprecipitation (Co-IP). Notably, non-POU domain-containing octamer-binding protein (NonO) was found to be a PRDM1 binding protein in the nucleus of MO-DCs. NonO is recruited to the PRDM1 binding site in the promoter region of IL-6. Knockdown of NonO expression by siRNA lessened suppression of IL-6 promoter activity by PRMD1 following LPS stimulation. While NonO binding to PRDM1 was observed in human myeloma cell lines, an effect of NonO on IL-6 expression was not observed. Thus, loss of NonO interrupted the inhibitory effect of PRDM1 on IL-6 expression in MO-DCs, but not plasma cells. Moreover, MO-DCs with low expression of PRDM1 or NonO induce an increased number of IL-21-producing T_{FH}-like cells *in vitro*. These data suggest that low level of PRDM1 and NonO lead to enhanced activation of MO-DCs and the regulation of MO-DC function by PRDM1 is mediated through cell lineage-specific mechanisms.

Keywords: PRDM1, NonO, IL-6, inflammation, dendritic cells

OPEN ACCESS

Edited by:
Femke Broere,
Utrecht University, Netherlands

Reviewed by:
Uma Sriram,
Lewis Katz School of Medicine at Temple University, United States
Attila Szabo,
University of Oslo, Norway
Deepika Sharma,
University of Chicago, United States

***Correspondence:**
Sun Jung Kim
SJKim@northwell.edu

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *05 February 2020*
Accepted: *03 June 2020*
Published: *10 July 2020*

Citation:
Lee K, Jang SH, Tian H and Kim SJ (2020) NonO Is a Novel Co-factor of PRDM1 and Regulates Inflammatory Response in Monocyte Derived-Dendritic Cells. Front. Immunol. 11:1436. doi: 10.3389/fimmu.2020.01436

INTRODUCTION

Positive regulatory domain 1 (PRDM1, also named BLIMP1) was identified as a repressor of interferon beta (IFN-β) gene expression in humans and mice (1, 2). PRDM1 is expressed in multiple cell lineages and is critical for early development (2–4). The immunological function of PRDM1 was first identified in B lymphocytes. Expression of PRDM1 is strongly induced in post-germinal center B cells committed to plasma cell (PC) differentiation (5, 6). In PCs, PRDM1 acts as a master transcription factor through positive regulation of genes involved in plasmablast (PB) and PC function, and the absence of PRDM1 in B cells in mice leads to a lack of PC with hypoimmunoglobulinemia despite normal B cell memory responses (7–9).

Genome-wide association studies (GWAS) have identified polymorphisms in *PRDM1* that are associated with autoimmune diseases. Single nucleotide polymorphisms (SNPs) predisposing to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are located in the intergenic

region between *PRDM1* and *ATG5* (10). Monocyte derived-dendritic cells (MO-DCs), but not B cells derived from healthy female individuals with the rs548234 SNP, which is a risk factor for SLE, show a lower level of *PRDM1* expression, suggesting that a proper expression of PRDM1 in dendritic cells (DCs) is required for immunological homeostasis in a gender-specific manner (11).

Immunoregulatory functions of PRDM1 in myeloid cells have been reported; mice with a DC-specific knockout of *Prdm1* (*Prdm1* CKO) spontaneously develop a lupus-like phenotype (11). Increased expression of the proinflammatory cytokine Interleukin-6 (IL-6) in DCs of *Prdm1* CKO mice, following Toll-like receptor (TLR) 4 stimulation, leads to an enhanced differentiation of follicular helper T cells (T_{FH}), revealing a potential pathogenic mechanism for *PRDM1* in autoimmune diseases (11). PRDM1 also participates in the process of antigen processing and presentation, and regulates expression of class II trans-activator (CIITA) in PCs and lymphocytes (12, 13), and cathepsin S (CTSS) in DCs (14). CTSS was higher in PRDM1-deficient DCs than in control DCs and increased CTSS activity contributes to development of autoantibodies and enhanced induction of T_{FH} cells in female *Pdrm1* CKO mice (14). In addition, PRDM1 was identified as a critical downstream regulator of the aryl hydrocarbon receptor (AHR) during MO-DC differentiation; a lack of AHR expression enhances monocytes to macrophages differentiation (15). These studies together suggest that PRDM1 mediates different regulatory functions in myeloid cells.

Studies in cell lines suggest that recruitment of chromatin regulators is important for the suppressive function of PRDM1 (16–19). Studies performed in primary lymphocytes showed that PRDM1 recruits cell-type specific co-factors in CD4+ T cells, CD8+ T cells, and in plasmablasts (20–22). While there are some common target genes among lymphocytes, the majority is cell type-dependent. These observations suggest that co-factors of PRDM1 are one of important contributor to cell-type dependent regulatory mechanisms of PRDM1. In this study, we identified co-factors of PRDM1 in MO-DCs by immunoprecipitation and mass spectrometry (IP-MS). Among the candidate proteins, a non-POU domain-containing octamer-binding protein (NonO, also named p54nrb) helps PRDM1 to suppress IL-6 expression by direct binding to the *IL6* promoter. Moreover, a deficiency of PRDM1 or NonO in MO-DCs increases differentiation of IL-21 producing T_{FH}-like cells. Together, these observations suggest that PRDM1 and NonO together regulate DC activation.

MATERIALS AND METHODS

Preparations of Peripheral Blood Mononuclear Cells (PBMCs) and MO-DCs Differentiation

The protocol for study of human blood was approved by the Institutional Review Board (approval number: 17-0075). PBMCs were purified from leukopack (NY Blood center) as described previously (14). To prepare MO-DCs, CD14+ monocytes were isolated from MO-DCs by EasySep Human CD14 positive selection kit II (StemCell Technologies) according to the manufacturer's protocol. CD14+ monocytes were cultured with RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S), 1% L-glutamine, 100 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech), and 50 ng/ml of recombinant human IL-4 (PeproTech) for 7 days. Cultures were kept at 37°C in a humidified atmosphere and 5% CO_2. On day 7, MO-DCs were collected only from the non-adherent cells and the purity of MO-DCs was confirmed by flow cytometry with antibodies which were purchased from eBioscience (anti-HLA-DR-FITC: LN3 and anti-CD209-PE/Cy7: eB-h209) (23). Over 85% of HLA-DR+CD209+ MO-DCs were obtained consistently. We excluded adherent cells since cells shows mixed population with CD209+ and CD209- with various degrees (**Figure S1A**).

Cell Lines

The HEK-293 cell line was purchased from ATCC (ATCC CRL-1573) and maintained in DMEM with 10% FBS, 1% P/S, and 1% L-glutamine. The human myeloma cell lines U-266, RPMI-8266 and Daudi (a gift from Dr. Chiorazzi, FIMR, NY) were maintained in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 15% FBS, 1% P/S, and 1% L-glutamine.

Co-IP and Mass Spectroscopy (MS) Assays

Co-IP was performed as described previously (24). Briefly, 2–5 μg of PRDM1 rabbit mAb (Cat# OAR03181, Aviva Systems Biology or cat# 9115s, Cell Signaling Technology) or normal rabbit immunoglobulin G (IgG) (Cat# 2729, Cell Signaling Technologies) were coupled to protein G or A-magnetic beads (DynaBead, Thermo Scientific). Anti-flag M2 magnetic beads (Milipore) were used to pull-down flag tagged RPDM1 in some experiments. Nuclear protein was extracted from PRDM1 transfected MO-DCs with a NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Scientific) and incubated with a bead-conjugated PRDM1 antibody or control IgG overnight at 4°C. The beads were washed and proteins bound by antibody were eluted by elution buffer and stored at −80°C until used for either immunoblotting or mass spectrometry. Mass spectrometry was performed, and analyses were done at cold spring harbor laboratory shared resources as described previously (25).

Immunoblotting

Western blot was performed as described (24). Cell extracts or eluted proteins were separated by 4–12% Bis-Tris polyacrylamide gel electrophoresis (PAGE) (Invitrogen). Proteins were transferred to polyvinyliden difluoride (PVDF) membrane (GE Amersham, Hybond-C or Millipore, Immobilon-FL) and blocked for 1 h at room temperature with 5% non-fat dry milk in TBS-T buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween20, pH 7.4). The membranes were then incubated with primary antibodies to HDAC1 (Cat# 5356s, Cell Signaling Technology), HDAC2 (Cat# 5113s, Cell signaling Technology), PRDM1 (Cat# 9115s), hnRNPM (Cat# SAB1404107, Sigma Aldrich), TP53BP1 (Cat# 4937s, Cell Signaling Technology), β-Actin (Cat# ab8226, Abcam), V5 (Cat# MA5-15253, Sigma Aldrich), and

Flag (Cat# F1804, Sigma Aldrich) overnight at 4°C. Proteins bound by antibody were visualized by ECL (Thermo Scientific, # 34580 or Advansta, K-12045) and sapphire biomolecular imager (Azure Biosystems).

Plasmids and Transient Transfections

Human *PRDM1* Tagged ORF Clone *PRDM1* (RC217363L1V) and human small interfering Ribonuclic acid (siRNA) oligo duplex exogenous (SR300437; *PRDM1* and SR321120; *NonO*) were purchased from Origene. *FLAG-NonO* (pCMV-myc-Flag-p54), *FLAG-TP53BP1* (pcDNA5-FRT/T0-Flag-53BP1), and *V5-hnRNPM* (pT7-V5-SBP-C1-HshnRNPM) expressing plasmids were purchased from Addgene. Transfections were prepared as described in previous study (24). For transient transfection to HEK-293 cells, 1–2 μg of plasmid was transfected to 70% confluent monolayered HEK-293 cells by Lipofectamin (Invitrogen). After 24 h, medium was replaced with complete medium and cells were further cultured for 2 days. 200 nM siRNA or 1–2 μg of plasmid was transfected to 10^6 MO-DCs at day 5 during differentiation by Human Dendritic Cell Nucleofector™ Kits (Lonza). After transfection, MO-DCs were further differentiated for 2 days and cells were harvested for experiments. 10^6 myeloma cells were transfected by using Nucleofector™ Kits (R kit for U-266, T kit for RPMI-8226, and V kit for Daudi) with 400 nM siRNA (Origene), according to manufacturer's instructions.

Proximity Ligation Assay (PLA) Assay

The *in situ* PLA was performed on fixed MO-DCs with Duolink *in situ* Detection Reagents Red (Sigma Aldrich) according to the manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde (PFA) at room temperature and washed with PBS. Samples were permeabilized with 0.5% Triton-X-100 in PBS and blocked by blocking solution (provided by the kit) for 1 h at 37°C. Primary antibodies against NonO (Cat# sc-376865, Santa Cruz), hnRNPM, TP53BP1, PRDM1, V5, Flag or normal rabbit IgG (Cat# 2729, Cell Signaling Technology) were incubated overnight at 4°C. The samples were washed twice for 5 min with buffer A (provided by the kit), followed by incubation with the PLA probes (Sigma Aldrich) for 60 min at 37°C. Subsequent ligation for 30 min at 37°C and amplification for 100 min at 37°C were performed. Finally, the samples were mounted using Duolink *in situ* Mounting Medium with DAPI (Sigma Aldrich). Z-Stacks Images were captured using a 60X oil objective on Zeiss Apotome 2 microscope and LSM 880 confocal microscopy (Carl Zeiss Microscopy). Three-dimensional foci counting analysis was performed with Imaris software (Imaris v8.0.2).

Chromatin Immunoprecipitation (ChIP) and PCR

ChIP assays were performed as previously described (14). 5 × 10^6 MO-DCs were cross-linked with 1% formaldehyde for 10 min at room temperature and quenched with 125 mM glycine. Cells were washed with ice-cold 1X DPBS twice. Cell pellets were lysed in 300 μl ChIP Lysis Buffer I (50 mM HEPES.KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100), ChIP Lysis Buffer II (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0), then ChIP Lysis Buffer III (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% *N*-lauroylsarcosine). All three lysis buffers were supplemented with complete proteinase inhibitor (Roche), and each lysis was performed for 10 min at 4°C with rotation. After lysis, chromatin was sheared by sonication (7 cycles of 30 s ON and 60 s OFF by Q500 sonicator) (Fisher), which generated fragments ranging from 200 to 800 bp. Ten percent Triton X-100 was added to sonicated chromatin (nuclear membrane and lipids were removed by centrifuge). Ten percent of sonicated chromatin supernatant was saved as input control. Sonicated chromatin was incubated with 2 μg of antibody-Protein G and A Dynabeads (Invitrogen) complex overnight at 4°C. Unbound chromatin was removed with RIPA Buffer (50 mM HEPES.KOH, pH 7.5, 100 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Sodium Deoxycholate), followed by one time washing with 10 mM pH 8.0 Tris elution buffer. Chromatin elution was done by incubation with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA 1% SDS) at 70°C for 10 min. DNA and chromatin de-crosslinking was done by incubation at 65°C for overnight in elution buffer. DNA elute was cleaned by PCR purification kit (Qiagen) and kept at −20°C until PCR or library prep for sequencing. To detect binding to *IL6* promoter regions, primer sets that detect each *PRDM1* consensus sequence was used for PCR. The PCR condition was as followed: 94°C for 5 min; 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 40 cycles.

Set1: F- 5′-GCCTCAATGACGACCTAAGC-3′, R- 5′-ACGT CCTTTAGCATGGCAAG-3′,
Set2: F- 5′-GCGATGGAGTCAGAGGAAAC-3′, R- 5′-AGCT GAAGTCATGCACGAAG-3′,
Set3: F- 5′-CCTGGAGACGCCTTGAAGTA-3′, R- 5′-CTGT GAGCGGCTGTTGTAGA-3′,
Set4: F- 5′-TACAGGGAGAGGGAGCGATA-3′, R- 5′-GGCA GAAAGGGGGAGAATAC-3′,
Set5: F- 5′-AAATGCCCAACAGAGGTCAC-3′, R- 5′-AAAC CAGACCCTTGCACAAC-3′,
Set6: F- 5′-CTCCCCCATTTTCATTTTCA-3′, R- 5′-TGGG GAAAGTGAGGTCATC-3′,
Set7: F-5′-TGAACATTTTATCATGAACACGAA-3′, R- 5′-CGTGCACTGTGATCCGTCTA-3′,
Set8: F- 5′-CGGTGAAGAATGGATGACCT-3′, R- 5′-GTGA CCTCTGTTGGGCATTT-3′.

Cloning IL-6 Promoter and Luciferase Reporter Assay

Primers to amplify the IL-6 area (forward, 5'-CGATATAG CCGAGCTGGAAG-3'; reverse, 5'- AAACCAGACCCTTGCA CAAC-3') yield 932-bp amplicon. The PCR condition was as followed: 94°C for 5 min; 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min 15 s for 30 cycles. *IL6* PCR product was cloned in pGL4.25 (Promega). 2 × 10^4 HEK-293 cells were plated in 12-well culture plates in DMEM containing 10% FBS and 1% penicillin-streptomycin. *IL6* promoter luciferase reporter construct and *tk-Renilla* luciferase construct was transfected

by Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, transfected cells were lysed and assayed for both firefly and Renilla luciferase activity using the Dual-GLO Luciferase Assay System (Promega). Luciferase activity was measured using a luminometer (Perkin Elmer Victor3). The relative luciferase activity was calculated by normalization to the level of Renilla luciferase.

T_{FH} Cell *in vitro* Differentiation

MO-DCs were differentiated and indicated siRNAs were transfected at day 5 during differentiation. Cells were further cultured for 2 days. MO-DCs were stimulated with lipopolysaccharides (LPS) (1.0 μg/ml) for 6 h and washed. Naïve CD4+ T cells were isolated from PBMCs by using an Easysep human naïve CD4+ T cell isolation kit (Stemcell technologies). 1.3×10^3 of LPS pre-stimulated MO-DCs or unstimulated MO-DCs were co-cultured with 4×10^4 naïve CD4+ T cells for 6 days. NC (negative control) was naïve CD4+ T cell alone and PC (positive control) was naïve CD4+ T cells with T_{FH} differentiation cocktails [CD2/3/28 activation beads (Miltenyl Biotec), IL-6 (50 ng/ml, R&D systems) and IL-12 (20 ng/ml, R&D systems)]. T_{FH} cells were analyzed by flow cytometry with a Fortessa (BD Biosciences). Fixable Viability Dye eFluor 506 (FVD, eBioscience) was used to exclude dead cells. For flow cytometry, antibodies were purchased from BioLegend (anti-CXCR5-APC: J252D4), eBioscience (anti-PD1-pacific blue: EH12.2H7 and anti-CD11c-Amcyan: B-ly6), and BD Bioscience (anti-IL-21-PE: 3A3-N2 and IFN-γ-APC/Cy7: B27).

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with Direct-zol RNA Micro Prep (Zymo Research,CA) and RNA samples were treated with DNase I to remove genomic Deoxyribonuclic acid (gDNA) contamination. cDNA was prepared with the iScript cDNA synthesis kit (Bio-Rad). Gene-specific primers were purchased from Taqman (Life Technologies) and qRT-PCR was performed with a Light cycler 480 II (Roche). Taqman primers: Hs00153368_m1 (*BCL6*), Hs00172187_m1 (*POLR2A*), Hs99999902_m1 (*RPLP0*), Hs00174131_m1 (*IL6*), Hs00153357_m1 (*PRDM1*), Hs00939763_g1 (*NonO*), Hs00175407_m1 (*CTSS*). Relative expression of a gene of interest to housekeeping gene was calculated by ΔCt or ΔΔCt.

ELISA

To measure the cytokine secretion, supernatants from the MO-DCs were collected and the level of IL-6 was measured by human IL-6 ELISA kit (DuoSet ELISA kit, R&D Systems, Minnesota, USA). The lower level of detection for the assay was 4.68 pg/ml.

Statistics

Statistical analysis was calculated and determined by a non-parametric Man-Whitney test in the Prism 6 (Graphpad software). $P < 0.05$ were considered significant.

RESULTS

NonO Is a PRDM1 Binding Protein in MO-DCs

PRDM1 is known to regulate gene expression by recruitment of chromatin modifiers, including histone deacetylases (HDACs), lysine-specific demethylase1 (LSD1), protein arginine methyltransferase (PRMT5), and euchromatic histone-lysine N-methyltransferase 2 (EHMT2, also known as G9a) in PCs and primordial germ cells (16–19). PRDM1 also recruits polycomb repressive complex 2 (PRC2) by directly binding the enhancer of zeste homolog 2 (*Ezh2*) domain of PRC2 in murine plasmablasts (26). We investigated whether PRDM1 recruits the same chromatin modifiers in MO-DCs. Binding of HDAC1, HDAC 2, PRMT5 or G9A to PRDM1 was assessed by Co-IP; however, no significant binding of any of those molecules to PRDM1 in MO-DCs was found (**Figure S1B** and data not shown).

To identify binding proteins of PRDM1 in a non-biased way, relative and absolute quantitation (iTRAQ) MS was performed on the nuclear fraction of MO-DCs immunoprecipitated by PRDM1 antibody. Compared to the fraction immunoprecipitated by control IgG, 39 proteins were pulled-down specifically by the anti-PRDM1 antibody (cutoff >1.5-fold) (**Table S1**). Consistent with the Co-IP data, there were no HDACs or other known chromatin modifiers identified by mass spectrometry. Thus, PRDM1 does not recruit detectable chromatin modifiers for regulation of target gene expression in MO-DCs.

Among the PRDM1-associated proteins identified by MS, we chose three molecules, NonO, Tumor Protein P53 Binding Protein 1(TP53BP1), and Heterogeneous Nuclear Ribonucleoprotein M (hnRNPM), as candidate co-regulators of PRDM1 due to their known transcriptional regulatory functions. To verify the interaction between those three proteins and PRDM1, Co-IP was performed in HEK-293 cells. Since HEK-293 cells do not express PRDM1 endogenously, they were transiently transfected with vectors encoding *PRDM1* and *Flag-NonO*, *Flag-TP53BP1*, or *V5-hnRNPM*. As expected, PRDM1 were not detected in input of non-transfected HEK-293 (**Figure 1A**, lane 1) and immunoprecipitated proteins from HEK-293 cell nuclear extract (PRDM1-negative) did not display any of the three proteins (NonO, TP53BP1, and hnRNPM) by Western blot (**Figure 1A**, lane 6). In contrast, anti-FLAG and anti-V5 immunoblotting, which detect Flag-NonO, Flag-TP53BP1, and V5-hnRNPM, showed an association between PRDM1 and NonO and hnRNPM in PRDM1-transfected cells (**Figure 1A**, lane 8 and 9) but no interaction between PRDM1 and TP53BP1 (**Figure 1A**, lane10).

PLA was used to verify these interactions in HEK-293 cells. PLA is an antibody-based detection technique that permits the assessment of colocalization between two proteins within < ∼40 nm distance in a cell (27, 28). PLA complexes are depicted as red puncta and each punctum represents an interaction between PRDM1 and a candidate molecule. PLA-positive red clusters were not detected in the technical control, which included incubation with only anti-PRDM1 antibody (**Figure 1B,** top panel). All three candidates, NonO, TP53BP1, and hnRNPM led to PLA

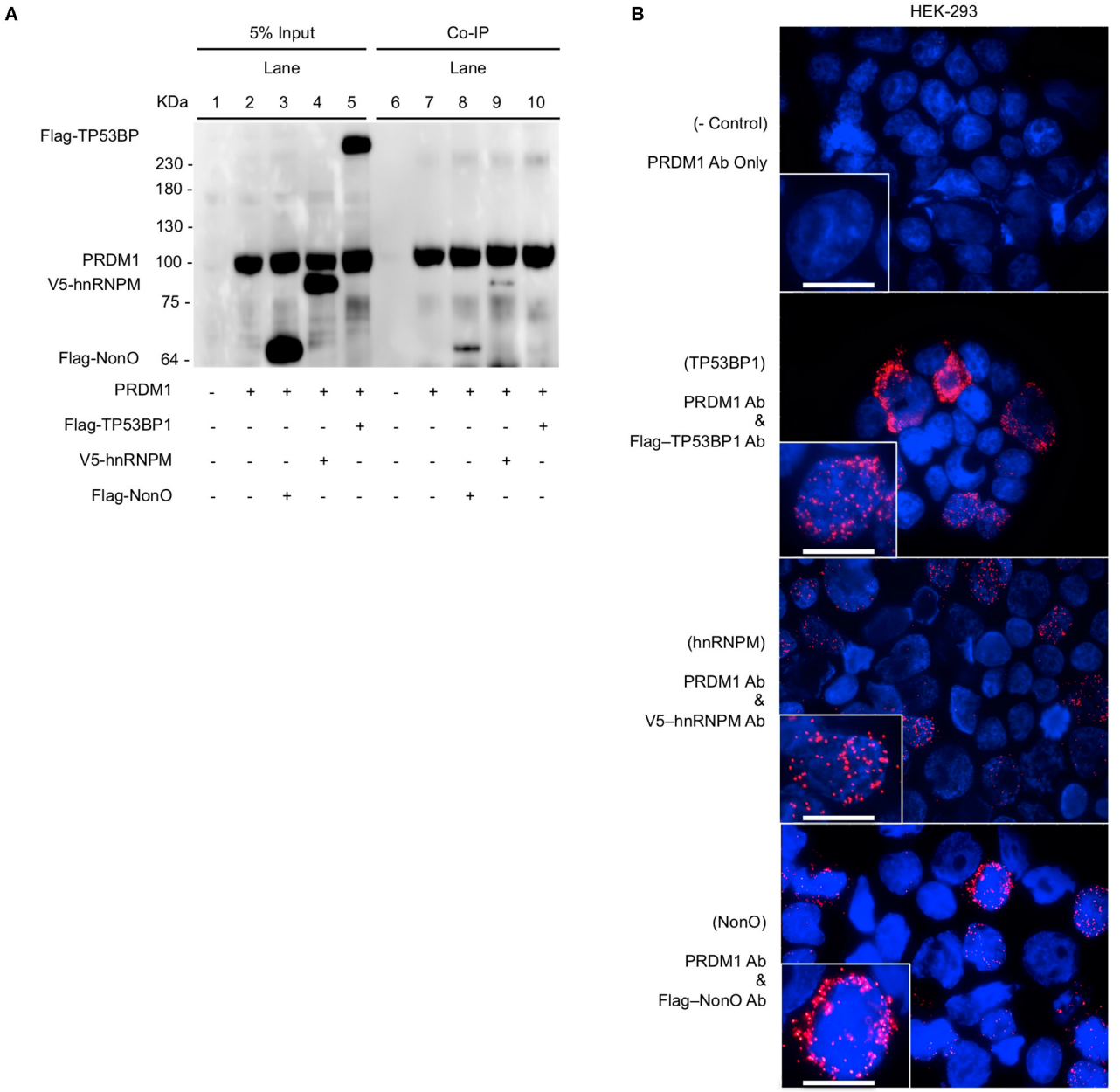


FIGURE 1 | Binding of PRDM1 and candidate proteins in HEK-293 cells. HEK-293 cells were transfected *PRDM1* alone or together with *Flag-NonO*, *Flag-TP53BP1*, or *V5-hnRNPM* expression vector. Binding between PRDM1 and each candidate proteins was detected by Co-IP and PLA. **(A)** Nuclear fraction was immunoprecipitated with anti- PRDM1 antibodies and immunoblotting was performed with anti-PRDM1, Flag-NonO, Flag-TP53BP1, or V5-hnRNPM antibody. A representative image from two independent experiments is shown. **(B)** Binding between PRDM1 and candidate proteins was visualized by PLA (red color) and nuclei were stained with DAPI (blue). Top panel; technical negative control PLA (PRDM1 antibody alone), other panels; detection of PLA (PRDM1 with Flag or V5 Ab). Scale bar = 10 μm. A representative image from three independent experiments. Co-IP, co-immunoprecipitation (Co-IP); PLA, proximity ligation assay.

positive clusters with PRDM1 (**Figure 1B**). The signals from hnRNPM and NonO were predominantly nuclear while signal from TP53BP1 was detected in the cytoplasm, suggesting that the interaction of hnRNPM and NonO with PRDM1 may be involved in regulation of gene expression while an interaction of TP53BP1 and PRDM1 may regulate pathways in the cytoplasmic compartment. This observation explains the lack of association of TP53BP1 and PRDM1 in the Co-IP of nuclear extracts.

We further validated these results in MO-DCs, in which we did not need to overexpress PRDM1. No PLA signals were detected in MO-DCs with any single primary antibodies and normal IgG (**Figure 2A,** left panel). As expected, PLA signals

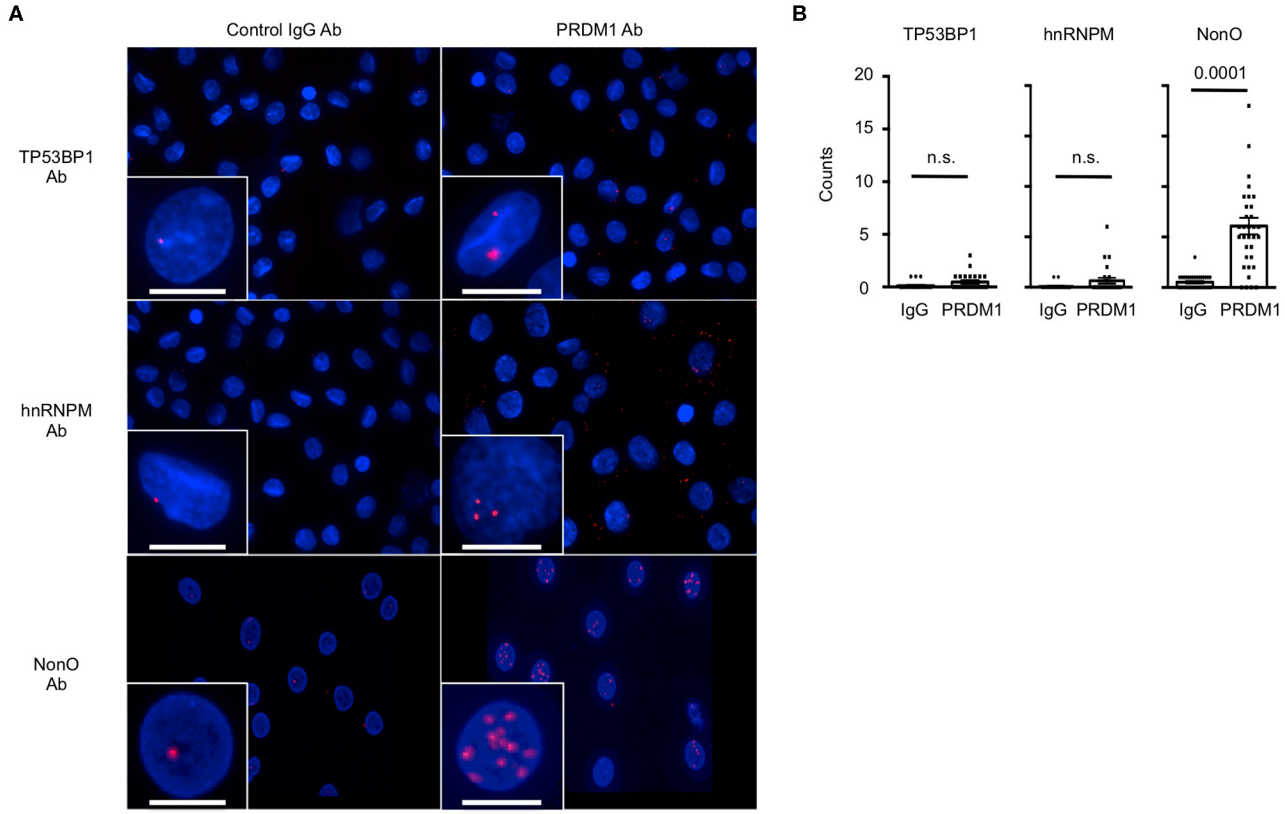


FIGURE 2 | The interaction of NonO and PRDM1 in the nucleus of MO-DCs. Binding of candidate proteins with PRDM1 in primary MO-DCs was measured by PLA. **(A)** MO-DCs were incubated with normal rabbit IgG (left column) or with anti-PRDM1 antibodies (right column) together with anti-TP53BP1, anti-hnRNPM, or anti-NonO antibodies. Their proximal interaction was assessed by PLA and visualized as red dots. Nucleus was stained with DAPI (blue). Each Z-Stacks maximum intensity projection image is a representative from three independent experiments and captured 60x magnification. Scale bar = 10 μm. **(B)** Quantification of the PLA signal on MO-DCs is represented. PLA signals in each nucleus were quantified by Imaris software. In the plot, horizontal bars indicate the mean with SEM and each dot represents counts of individual nucleus (n = 40–50). Significance determined by unpaired t-test.

were detected between PRDM1 and NonO in the nucleus of MO-DCs (**Figure 2A,** the bottom of right panel). There was no significant signal detected with hnRNPM and TP53BP1 in either the nucleus or the cytoplasm (**Figure 2A,** the top and middle of right panel). Quantitative analysis of the PLA signal between PRDM1-candidate proteins and negative control-IgG confirmed specific PLA signals between PRDM1 and NonO (**Figure 2B**, right graph).

Additionally, PRDM1 in nuclear extracts coprecipitated with NonO but not with hnRNPM and TP53BP1 (**Figure S2**). These inconsistent results obtained from primary MO-DCs and HEK-293 cells are likely due to the overexpression of PRDM1 in HEK293 cells. The data obtained from MS, Co-IP and PLA confirmed that NonO is a novel PRDM1 binding protein in the nucleus of MO-DCs.

NonO Co-regulates Expression of IL-6 in MO-DCs

Knowing that PRDM1 and NonO interact in the nucleus of MO-DCs, we further investigated whether NonO participates in the transcriptional function of PRDM1. Previous data showed that the level of the proinflammatory cytokine IL-6 was negatively regulated by PRDM1 in DCs in response to LPS stimulation (11, 14, 29). If NonO is required for PRDM1-mediated suppression of target gene expression, NonO-deficiency could lead to an increase in the level of IL-6 after LPS stimulation. We confirmed the binding of PRDM1 and NonO in MO-DCs after LPS stimulation (**Figure 3A**). Next, *NonO* or *PRDM1* targeting siRNA or scrambled control siRNA was transfected into MO-DCs and the level of *IL-6* was measured. Effective knockdown of NonO, PRDM1 or both was achieved; about 50% of either *NonO* or *PRDM1* mRNA was present in *NonO, PRDM1* or both *NonO and PRDM1*-siRNA compared to control siRNA transfected MO-DCs (**Figure 3B**). *NonO* expression was unchanged in PRDM1-deficient MO-DCs and PRDM1 expression was unchanged in NonO-deficient MO-DCs. To investigate whether *IL-6* expression is regulated by the level of NonO, PRDM1 or both, the level of *IL-6* was measured in the basal state and at 6 h after LPS stimulation. The basal level of *IL-6* mRNA and IL-6 protein in the supernatant were minimal and no change was detected with knock down of NonO, PRDM1, or both (**Figure S3**). In contrast, following LPS

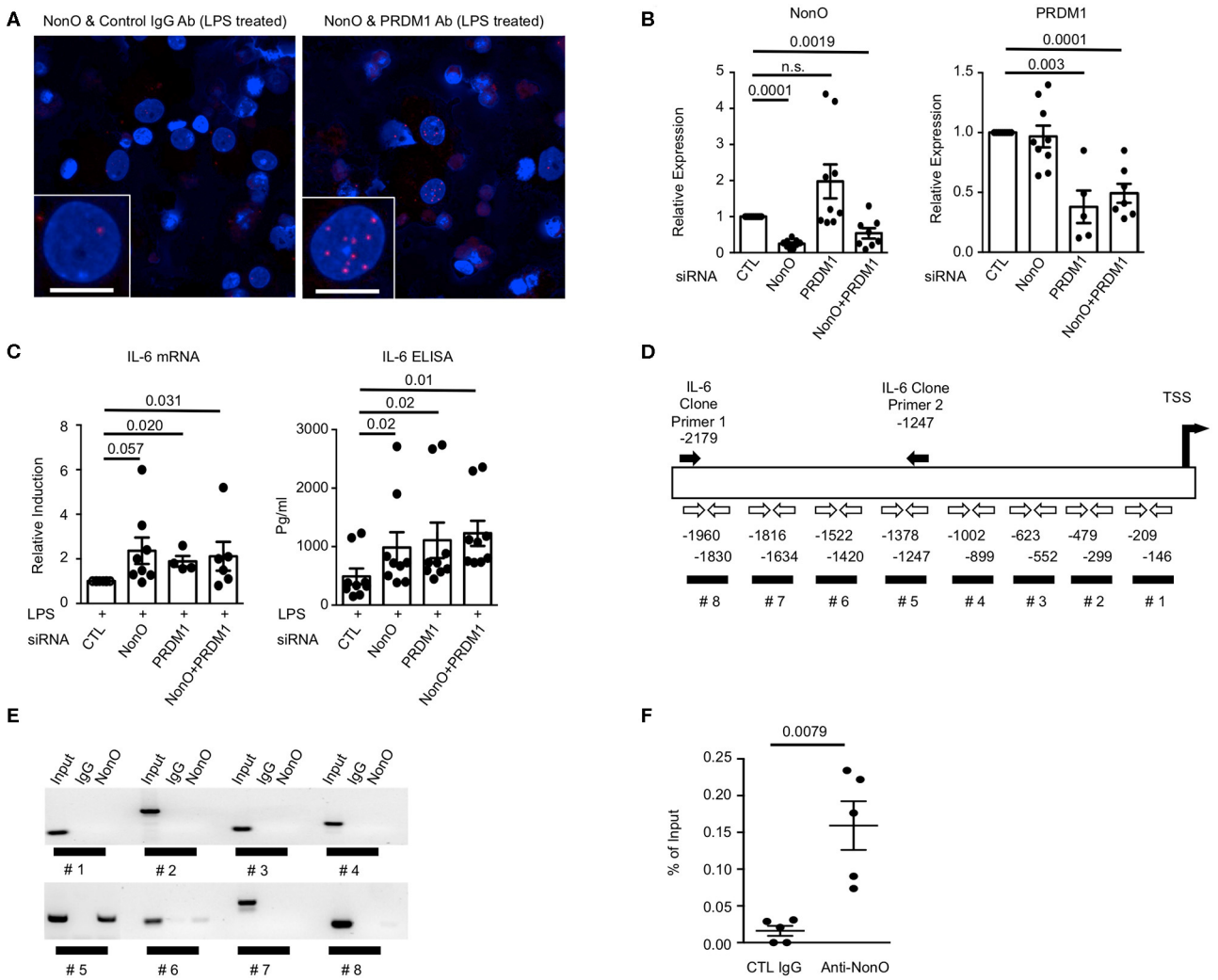


FIGURE 3 | NonO-dependent regulation of IL-6 by PRDM1 in MO-DCs. **(A)** MO-DCs were stimulated with LPS for 6 h and stained with normal rabbit IgG + anti-NonO antibodies (left column) or with anti-PRDM1 antibodies + anti-NonO antibodies (right column). Their proximal interaction was assessed by PLA and visualized as red dots. Nucleus was stained with DAPI (blue). Each Z-Stacks maximum intensity projection image is a representative from three independent experiments and captured 60x magnification. Scale bar = 10 μm. **(B)** To knock down NonO, PRDM1, and both (NonO and PRDM1) expression, indicated siRNA or control siRNA was transfected into MO-DCs and NonO and PRDM1 expression level were quantified by qRT-PCR. Bar graph is a mean ± SEM ($n = 9$). Significance determined by Man-Whitney test. **(C)** Indicated siRNA or control siRNA transfected MO-DCs were cultured with or without LPS (1 μg/ml) for 6 h, and total RNA was purified. Level of *IL6* was measured by qRT-PCR and relative induction was calculated by normalization to the level of LPS stimulated control. Supernatant concentrations of IL-6 obtained from the cultures were measured using enzyme-linked immunosorbent assay (ELISA). Bar graph is a mean ± SEM ($n = 9$). Significance determined by Mann Whitney test. **(D)** Diagram of human *IL6* promoter region with indication of putative PRDM1 binding site (black bar #1–#8) and PCR primers (open arrow). Primer set for *IL6* promoter cloning is designated as black arrows. ChIP was performed with anti-NonO antibodies or control IgG from MO-DCs. **(E)** PCR result was visualized in agarose gel. Binding of NonO to PRDM1 consensus sequences within the *IL6* promoter were assessed by each primer set (indicated in **C**). **(F)** To quantify the binding of NonO to #5 region, qPCR was performed and calculated by the percent of input. The graph is a mean ± SEM ($n = 5$). Significance determined by Mann Whitney test.

stimulation, IL-6 induction (both transcripts and protein in the supernatant) was increased in siRNA-transfected MO-DCs with NonO, PRDM1, or both compared to control siRNA-transfected MO-DCs (**Figure 3C**). There was no synergistic effect observed in double-knock down MO-DCs, suggesting PRDM1 and NonO are in a same regulatory pathway. These data demonstrate that NonO-deficiency and PRDM1-deficiency lead to the up-regulation of IL-6 in LPS stimulated MO-DCs.

Previous reports demonstrated that NonO can regulate gene expression by binding to promoter regions (transcriptional regulation) or by binding to mRNA (post-transcriptional regulation) (30–32). Therefore, we investigated the mechanism of NonO-mediated IL-6 expression in MO-DCs. First, binding of NonO to PRDM1-binding sites in the *IL6* promoter area was investigated. A search for the PRDM1 binding motif in the *IL6* promoter area revealed

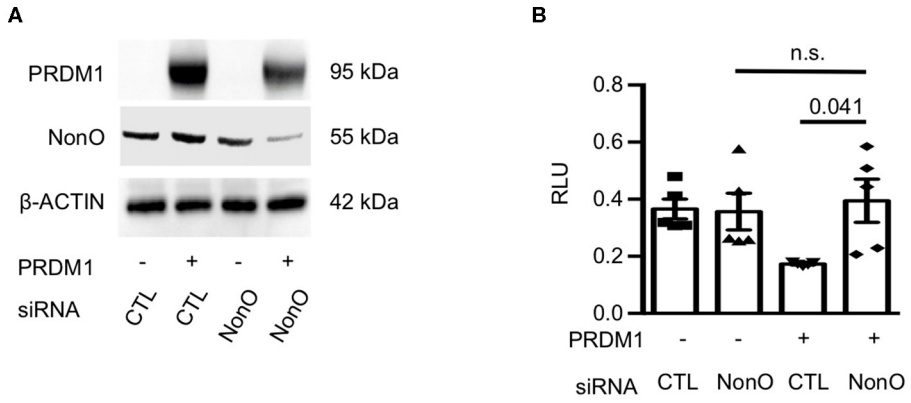


FIGURE 4 | NonO regulates IL-6 promoter activity. **(A)** HEK-293 cells were transfected with NonO siRNA or *PRDM1* expressing plasmid. The knockdown efficiency of siRNA and PRDM1 level was verified by immunoblott analysis. β- actin was used as a loading control. Gel image is a representative from two independent experiments. **(B)** The *IL6* promoter activity under indicated conditions was determined by luciferase reporter gene analysis. pGL4.25 were used as control vectors. Mean values of relative luciferase unit (RLU; normalized on Renilla luciferase) from three independent experiments. Bar is a mean ± SEM ($n = 4$). Significance determined by unpaired *t*-test.

eight potential PRDM1 binding sites which contain the consensus sequence (A/C)AG(T/C)GAAAG(T/C)(G/T) or (A/C)AG(T/C)GAAAT(T/C)(G/T) within 2,000 bp upstream from transcription start site (TSS) (33) (**Figure 3D**, #1–#8). We first performed ChIP-PCR using anti-NonO antibody or control antibody and the binding of NonO to PRDM1 binding sites in the *IL6* promoter region was assessed. The ChIP efficiency was optimized by detection of *P4Hα1*, a known target gene of NonO in DNA precipitated with anti-NonO antibody compared to control IgG (data not shown) (32). We performed PCR analysis with primer sets at multiple sites throughout the IL-6 gene; region #5 (−1,247∼–1,378 bp TSS) was significantly enriched in DNA precipitated with anti-NonO antibody (**Figure 3E**). Thus, the #5 region is recognized by NonO. The percent of input (%IP) was calculated from the quantitative PCR (qPCR) and 2–5 fold more enrichment was observed with anti-NonO antibody compared to control IgG (**Figure 3F**). To confirm whether PRDM1 can bind to the same recognition sequence as NONO, we performed ChIP with anti PRDM1 antibody. We could detect enrichment of PRDM1-binding to IL-6 promoter at region (#5), but the difference between control IgG and PRDM1 was not significant (**Figure S4**). Taken together, NonO-PRDM1 complexes are recruited to *IL6* promoter region to suppress IL-6 expression in MO-DCs.

To further investigate whether NonO can regulate the transcription of *IL6,* a luciferase reporter assay was performed. Since ChIP-PCR results showed that NonO/PRDM1 binding was enriched in the proximal region [−1.3∼–2.2 kb] of the *IL6* gene promoter, we engineered an *IL6* promoter-Luc plasmid (pGL4.25) containing NonO/PRDM1-binding region of human genomic DNA. To modulate the level of PRDM1 and NonO in the HEK-293 cell line, HEK-293 cells were transfected with a PRDM1 expressing plasmid with control siRNA or with *NonO* siRNA. *NonO* siRNA led to a 30–60% decrease in NonO protein levels compared to levels in control siRNA transfected HEK-293 cells (**Figure 4A**). As expected, PRDM1 suppressed *IL6* promoter activity; this suppressive effect was abrogated by a decrease in NonO (**Figure 4B**). These results demonstrate that NonO functions to enable transcriptional repressor of the *IL6* gene by PRDM1. There is no significant change in *IL6* promoter activity by NonO deficiency without PRDM1 expression; thus, the regulatory mechanism of NonO depends on the PRDM1 expression level.

NonO-PRDM1 Complexes Regulate the Generation of T_{FH}-Like Cells

IL-6 production is one of key factors for murine follicular helper T cell (T_{FH}) differentiation, and an increased production of IL-6 in DCs leads to an expansion of T_{FH} in *Prdm1* CKO mice (11, 14, 29). To address the function of PRDM1-deficiency and NonO-deficiency in DCs on the differentiation of $CD4^+$ T_{FH} cells, unstimulated or LPS pre-stimulated MO-DCs were co-cultured with naïve CD4+ T cells. After co-culture, surface phenotype and cytokine production by T cells were investigated by flow cytometry (**Figure 5A**). After co-culture, live CD4+ T cells were identified by exclusion of FVD-positive (dead cells) and CD11c-positive (MO-DCs). There was no difference in the expansion of T cells, and CXCR5-positive T cells were not strongly induced in any culture condition (data not shown). Interestingly, LPS-stimulated PRDM1-deficient or NonO-deficient MO-DCs induced a higher percent of IL-21+CXCR5-PD1+ cells compared to control siRNA-treated MO-DCs (**Figure 5B**). NonO-deficient MO-DCs also induced higher percent of IL-21+CXCR5-PD1+ T cells even in the absence of LPS stimulation, but this effect was not observed in PRDM1- or double deficient MO-DCs (**Figure 5B**). We do not

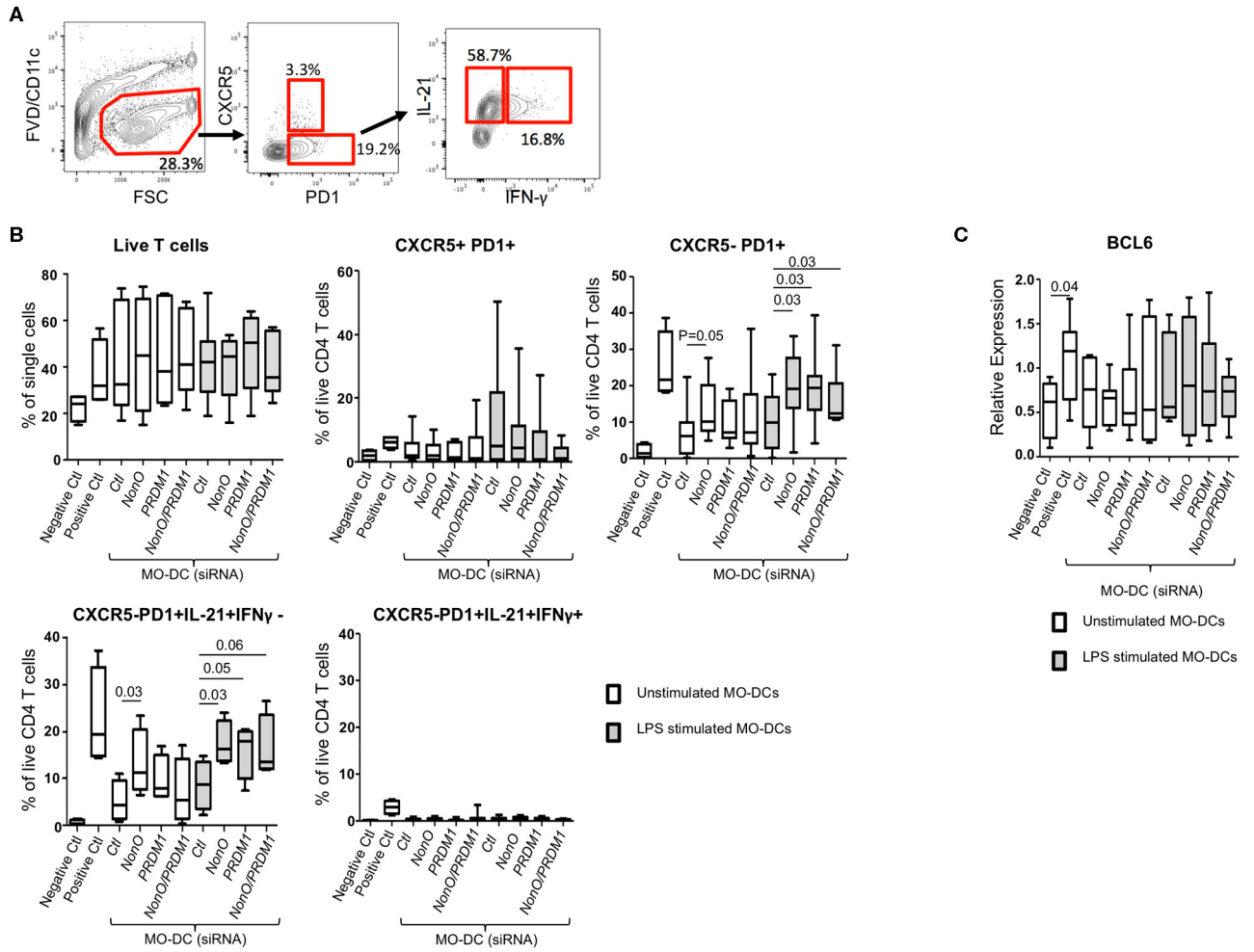


FIGURE 5 | PRDM1- or NonO-deficient MO-DCs induce increased T_{FH} differentiation. Allogenic culture of naïve CD4+ T cells and MO-DCs was set up to induce T_{FH} differentiation. Expression levels of PRDM1 or NONO in MO-DCs were modulated by transfection with siRNAs before co-culture. After 6-days culture, T_{FH} cell differentiation was measured by IL-21, IFNγ, CXCR5, and PD1 expression by flow cytometry. **(A)** A representative flow image. **(B)** Dead cells were excluded using Fixable Viability Dye eFluor 506, and the percentage of live $CD4^+$ T cells was calculated. T_{FH} -like cells (CXCR5+/PD1+), CXCR5- helper T cells (CXCR5-/PD1+), and cytokine expressing helper T cells (CXCR5-/PD1+/ IL21+/ IFN-γ-, and CXCR5-/PD1+/IL21+/ IFN-γ+) were calculated and plotted. Co-culture of T cells with LPS pre-stimulated MO-DCs was indicated with gray filled box and culture with unstipulated MO-DCs was indicated with open box. Negative control is naïve CD4+ T cell alone and Positive control is naïve CD4+ T cells with CD2/3/28 activation beads IL-6 (50 ng/ml) and IL-12 (20 ng/ml). In the Box-and-Whisker plot, horizontal bars indicate the median, boxes indicate 25–75th percentile, and the whiskers indicate 10 and 90th percentile. Four independent experiments ($n = 9$). Significance determined by Mann Whitney test. **(C)** *BCL6* expression was quantified by qRT-PCR. Relative expression was calculated to the level of housekeeping gene, *POLR2A*. In the Box-and-Whisker plot, horizontal bars indicate the median, boxes indicate 25–75th percentile, and the whiskers indicate 10 and 90th percentile. Three independent experiments ($n = 9$). Significance determined by Mann Whitney test.

know the mechanism, but NonO may regulate other regulatory molecules which positively regulate T cell differentiation or survival. We also compared IFN-γ production in T cells, but none of T cells were IFN-γ-positive (**Figure 5B**). To confirm the IL-21-producing T cells are T_{FH} cells, we measured the induction of B-Cell Lymphoma 6 (BCL6) which is a master transcription factor of Tfh cells (34). The *BCL6* induction was small and not significantly higher than negative control group, and there was no difference of *BCL6* levels among the groups (**Figure 5C**). Therefore, NonO, PRDM1, or PRDM1/ NonO deficient MO-DCs generate more IL-21 producing T_{FH}-like cells. No synergistic effects of PRDM1 and NonO were observed in IL-21 producing T_{FH}-like cell differentiation. Increased production of IL-6 might contribute to this alteration.

NonO-PRDM1 Interaction Does Not Regulate IL-6 Expression in Myeloma Cells

Since plasma cells express a high level of PRDM1 and secrete IL-6, we wanted to know whether PRDM1 and NonO regulate IL-6 in the human myeloma B cell lines, U-266, and RPMI-8226 since both cell lines express a high level of PRDM1 and NonO (**Figure S5A**). Daudi, a non-myeloma B cell line

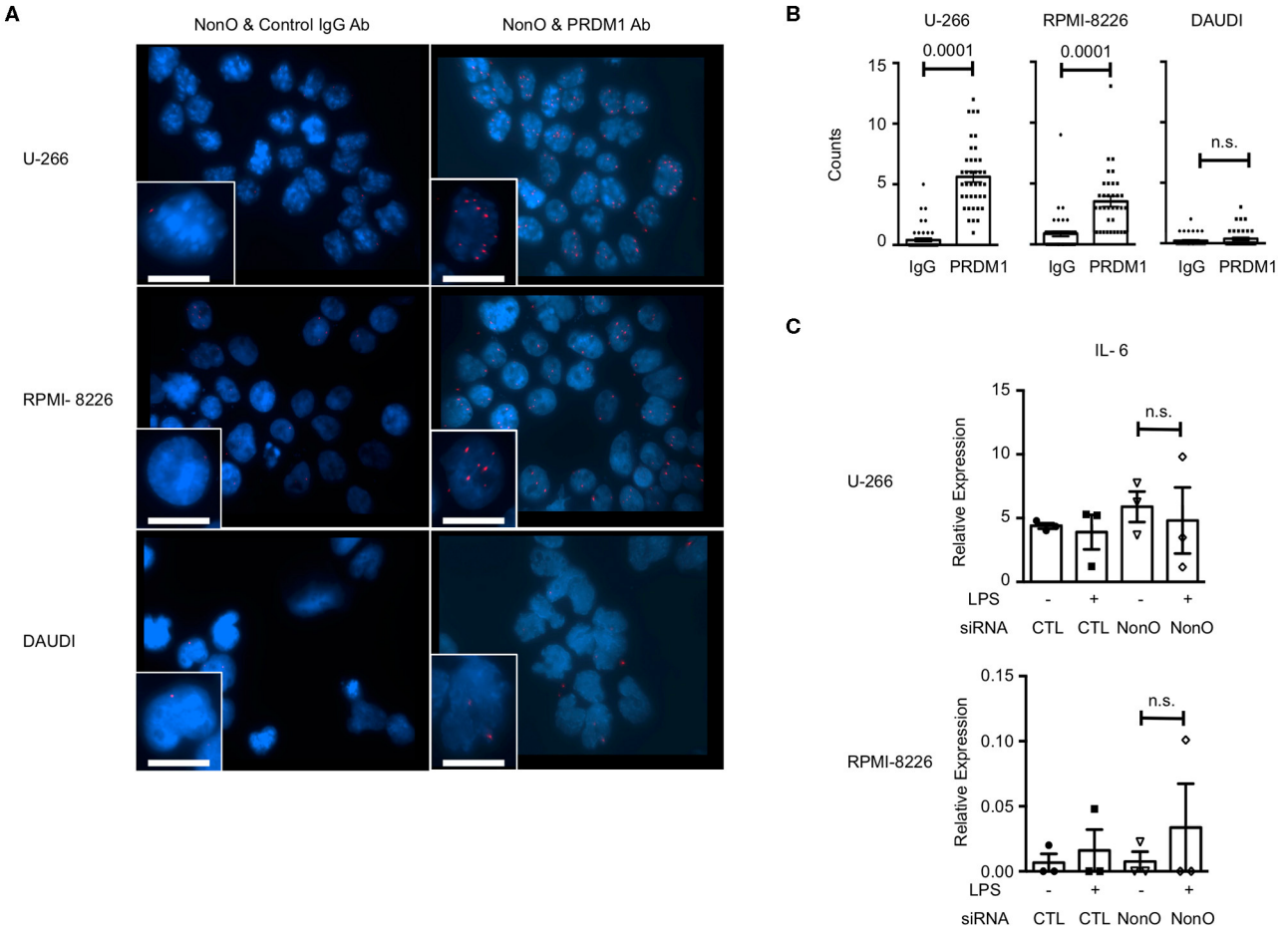


FIGURE 6 | NonO binds with PRDM1 but does not regulate IL-6 in myeloma cells. **(A)** Binding between PRDM1 and NonO in human myeloma cells was visualized by PLA (red color) and nuclei were stained with DAPI (blue). Left columns are control groups of PLA (anti-NonO and control IgG) and right columns are experimental groups (anti-NonO and anti-PRDM1). Scale bar = 15 μm. Images were taken at 60x magnification. A representative image from three independent experiments. **(B)** PLA signals in each nucleus were quantified by Imaris software (n = 30–40). Significance determined by Mann Whitney test. **(C)** 48 h *NonO* or control siRNA post transfection, cells were cultured with or without LPS (1 μg/ml) for last 6 h and level of *IL-6* measured by qRT-PCR. Bar graph is a mean ± SEM (n = 3). Significance determined by unpaired t-test.

with a high level of NonO but not PRDM1, was used as a negative control. Using the PLA assay, we found a NonO and PRDM1 interaction in U-266 and RPMI-8226 in nucleus but not in Daudi (**Figures 6A,B**). Next, we tested whether NonO participates in PRDM1-mediated IL-6 production in these cells. NonO knockdown mediated by siRNA was sufficient to decrease the NonO expression level in both U-266 and RPMI-8226 cells (**Figure S5B**). In contrast to MO-DCs, there is high level of endogenous IL-6 expression in myeloma cells which was not further increased by stimulation with LPS, and no significant induction of IL-6 when NonO levels were decreased (**Figure 6C**). Similarly, PRDM1-deficiency did not increase the expression level of basal or LPS stimulated *IL-6* mRNAs in U-266 (**Figure S5B**). Hence, in myeloma cells, NonO could be recruited to a PRDM1 complex but no PRDM1-mediated regulatory effects on IL-6 expression by NonO-deficiency and PRDM1-deficiency were observed.

DISCUSSION

PRDM1 is a transcription factor that is expressed in multiple immune cells including myeloid cells (22, 35, 36). A proper expression is required for terminal differentiation of myeloid lineage cells (36). It is also involved in immune homeostasis and an insufficient level of PRDM1 in DCs leads to a breakdown in immune tolerance in mice (11). However, little is known about the molecular mechanisms behind its function in myeloid cells. Previous reports suggest that the suppressive function of PRDM1 depends on its cofactors. In order to identify cofactors of PRDM1 in MO-DCs, we performed both Co-IP and MS experiments. Binding of putative cofactors to PRDM1 was assessed by PLA. These results show that a PRDM1-NonO interaction occurs which is localized to the nucleus. The interaction can be observed even after LPS stimulation. Together, these molecules regulate IL-6 expression. The regulatory function

on IL-6 is observed only in MO-DCs but not in human myeloma cells. It is well-accepted that IL-6 positively regulates IL-21 production in T cells, and the activation of STAT3 which is downstream of IL-6R signaling pathway is required for transcriptional activation of IL-21 (37–39). Deficiency of either NonO or PRDM1 in MO-DCs also leads to an expansion of IL-21-producing T_{FH}-like cells *in vitro*, suggesting that a proper expression of both NonO and PRDM1 is required for the proinflammatory function of MO-DCs. Interestingly, NonO might regulate function of MO-DCs which induces helper T cell differentiation without LPS stimulation. This alteration is not by IL-6 expression. Indeed, NonO itself can regulate gene expression in multiple mechanisms, including chromatin remodeling, transcriptional regulation, or post-transcriptional regulation (30, 40). We do not know what molecules are targeted by NonO in MO-DCs under homeostatic condition, and this needs to be investigated.

It has been previously shown by us and others that allele specific effects on gene expression may be cell lineage specific. This has been noted to relate to polymorphism-generated acquisition or loss of transcription factor binding sites. Indeed, a PRDM1 SNP that is associated with increased risk for developing SLE is operative in myeloid cells. The risk allele has a KLF4 binding site, which leads to decreased gene expression. As B cells do not express KLF4, there is no allele-specific change in expression of PRDM1 in B cells (23). Here we show differential effects of transcription factors in myeloid cell and B cells, even when both lineages express the transcription factors and the target gene. The mechanism for this requires elucidation. This could result from different chromatin accessibility of target genes, from multi-protein complexes including other unidentified transcription factors or from inhibitors of transcription factor binding to some regulatory regions of the target gene. In our case, the difference is not due to the accessibility of a PRDM1-NonO complex to the genomic area since both cells readily express IL-6. The functional difference may depend on additional cell type-specific co-regulators in myeloid cells and B cells. Indeed, NonO is known to be present in multi-protein complexes in the nucleus. In an *in vitro* system, PU.1 (Spi-1) binds to NonO and impedes NonO binding to RNA (41); the same may relate to DNA binding although it was not studied in that report. PU.1 is highly expressed in myeloid cells and in early stage of B cells, but its expression is suppressed in plasma cells (42) and completely negative in myeloma cells (43). Therefore, PU.1 might be a candidate regulator in regulation of function of PRDM1/NonO complex.

Additional remaining questions are what genes other than IL-6 are regulated by PRDM1 in conjunction with NonO and what genes are regulated by PRDM1 independent of NonO. CTSS is another gene which is negatively regulated by PRDM1 in MO-DCs (14), but CTSS expression was not changed by knock down of NonO (**Figure S6**). This suggests PRDM1 regulates gene expression in both a NonO-dependent and NonO-independent manner in the same cell, with CTSS as one example of NonO-independent regulation of PRDM1. All of these questions are critical for understanding the mechanism of gene regulation by PRDM1 and function in MO-DCs.

In summary, our data demonstrated that NonO is a co-factor of PRDM1 and recruitment of NonO by PRDM1 is required for transcriptional regulatory function of PRDM1 in MO-DCs. The absence of PRDM1 or NonO increased the expression of IL-6 which is a positive regulator of IL-21-producing T_{FH}-like cell differentiation. NonO interaction to PRDM1 regulates gene transcription in MO-DCs but not in myeloma cells, representing a new paradigm for exploring lineage specific effects of transcriptional regulators.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SK designed and conceived the study and analyzed and interpreted the data. SJ performed the experiment of Co-IP and mass spec and helped analyze the data. KL performed most experiments, data analysis, and helped interpret the data. HT performed gene expression studies and cell culture. KL and SK wrote the manuscript. All contributing authors have agreed to the submission of this manuscript for publication.

FUNDING

This work was supported by a grant from the US National Institute of Health (R01 AR065209 to SK, SJ, HT, and KL).

ACKNOWLEDGMENTS

We are especially thankful to B. Diamond for critiques on manuscript writing, B. Volpe and E. Chang at the FIMR for generous support and technical help of microscope imaging and quantification.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01436/full#supplementary-material

Figure S1 | (A) A representative flow image of MO-DCs. Day 7 of MO-DCs (non-adherent cells) and adherent cells were collected and viability and the purity were investigated by exclusion of live/dead fixable marker, FVD and HLA-DR and CD209 antibodies. Purity of MO-DC was calculated by percentage of live HLA-DR/CD209 double positivity. **(B)** Assessment of HDAC binding to PRDM1 by Co-IP. Nuclear fraction of MO-DCs was immunoprecipitated with anti- PRDM1 antibodies or control IgG and immunoblotting was performed with anti-HDAC1 or anti-HDAC2 antibodies. A representative image from two independent experiments is shown.

Figure S2 | Binding of PRDM1 with NonO, hnRNPM, and TP53BP1 by Co-IP. Nuclear fraction of MO-DCs were incubated with ant-PRDM1 or control IgG and immunoblotting was performed with anti-NonO, hnRNPM, or TP53BP1 antibodies. Input is an unfractionated total nuclear extract. A representative image of two independent experiments.

Figure S3 | The basal level of *IL-6* transcript and protein were measured by qRT-PCR and ELISA. NonO, PRDM1, and both (NonO and PRDM1) siRNA or

control siRNA transfected MO-DCs were cultured without LPS (1 μg/ml) for 6 h, and total RNA was purified. Relative level of *IL-6* was measured by qRT-PCR and normalized to the level of housekeeping gene, *POLR2A*. Supernatant concentrations of IL-6 obtained from the cultures were measured using ELISA. Bar graph is a mean ± SEM ($n = 9$). Significance determined by Mann Whitney test.

Figure S4 | Assessment of PRDM1 binding to *IL6* promoter regions by ChIP-qPCR. To test PRDM1 binding to *IL6* promoter, ChIP was performed. Nuclear fraction of MO-DCs and ChIP was performed by anti-RPDM1 or control IgG as described in material and method. PCR **(A)** or qPCR **(B)** was performed to assess binding of PRDM1 by primers described in material methods. #1–#8 indicates each region including putative PRDM1 binding sites in IL6 promoter. **(A)** is a representative image of three independent experiments. **(B)** To quantify the binding of PRDM1 to #5 region, qPCR was performed and calculated by the percent of input. Each dot represents an individual sample and the bar represents the mean ±SEM ($n = 3$). Significance determined by Mann Whitney test.

Figure S5 | Expression of *IL6* by NonO or PRDM1 in myeloma cells. **(A)** NonO expression was knock down by transfection of anti-NonO siRNA or scrambled control siRNA. After transfection, relative level of *NonO* was measured by qRT-PCR and normalized to the level of housekeeping gene, *POLR2A*. **(B)** To knock down the PRDM1 expression, anti-*PRDM1* siRNA, or control siRNA was transfected to U266 cells and *PRDM1* level was measured by qRT-PCR. U266 cells transfected with control or anti-PRDM1 siRNA was cultured with or without LPS (40 μg/ml) for 6 h. Relative level of *PRDM1*, *IL6* was normalized to the level of *POLR2A*. Each dot represents an individual sample and the bar is the mean ± SEM. Significance determined by Mann Whitney test.

Figure S6 | The level of *CTSS* was measured by qRT-PCR. NonO, PRDM1, and both (NonO and PRDM1) siRNA or control siRNA transfected MO-DCs were cultured with or without LPS (1 μg/ml) for 6 h, and total RNA was purified. Relative level of *CTSS* was measured by qRT-PCR and normalized to the level of housekeeping gene, *RPLP0*. Then, relative induction was calculated by normalization to the level of control. Bar graph is a mean ± SEM ($n = 6$). Significance determined by Mann Whitney test.

Table S1 | Mass spectrometric identification of candidate PRDM1 binding proteins in MO-DCs. The comparative analysis of peptide and protein quantification in normal IgG and PRDM1 of PRDM1-sufficient MO-DCs are subjected through iTRAQ-based quantitative proteomics with cutoff >1.5-fold. The experiment was repeated two times. iTRAQ, isobaric tags for relative and absolute quantitation; MO-DCs, monocyte derived-dendritic cells.

REFERENCES

1. Keller AD, Maniatis T. Identification and characterization of a novel repressor of beta-interferon gene expression. *Genes Dev.* (1991) 5:868–79. doi: 10.1101/gad.5.5.868
2. Keller AD, Maniatis T. Only two of the five zinc fingers of the eukaryotic transcriptional repressor PRDI-BF1 are required for sequence-specific DNA binding. *Mol Cell Biol.* (1992) 12:1940–9. doi: 10.1128/MCB.12.5.1940
3. Fairfax KA, Corcoran LM, Pridans C, Huntington ND, Kallies A, Nutt SL, et al. Different kinetics of blimp-1 induction in B cell subsets revealed by reporter gene. *J Immunol.* (2007) 178:4104–11. doi: 10.4049/jimmunol.178.7.4104
4. Chang DH, Cattoretti G, Calame KL. The dynamic expression pattern of B lymphocyte induced maturation protein-1 (Blimp-1) during mouse embryonic development. *Mech Dev.* (2002) 117:305–9. doi: 10.1016/S0925-4773(02)00189-2
5. Robertson EJ, Charatsi I, Joyner CJ, Koonce CH, Morgan M, Islam A, et al. Blimp1 regulates development of the posterior forelimb, caudal pharyngeal arches, heart and sensory vibrissae in mice. *Development.* (2007) 134:4335–45. doi: 10.1242/dev.012047
6. Angelin-Duclos C, Cattoretti G, Lin KI, Calame K. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression *in vivo*. *J Immunol.* (2000) 165:5462–71. doi: 10.4049/jimmunol.165.10.5462
7. Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity.* (2002) 17:51–62. doi: 10.1016/S1074-7613(02)00335-7
8. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity.* (2003) 19:607–20. doi: 10.1016/S1074-7613(03)00267-X
9. Turner CA, Mack DH, Davis MM. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B-lymphocytes into immunoglobulin-secreting cells. *Cell.* (1994) 77:297–306. doi: 10.1016/0092-8674(94)90321-2
10. Zhou XJ, Lu XL, Lv JC, Yang HZ, Qin LX, Zhao MH, et al. Genetic association of PRDM1-ATG5 intergenic region and autophagy with systemic lupus erythematosus in a Chinese population. *Ann Rheum Dis.* (2011) 70:1330–7. doi: 10.1136/ard.2010.140111
11. Kim SJ, Zou YR, Goldstein J, Reizis B, Diamond B. Tolerogenic function of Blimp-1 in dendritic cells. *J Exp Med.* (2011) 208:2193–9. doi: 10.1084/jem.20110658
12. Smith MA, Wright G, Wu J, Tailor P, Ozato K, Chen X, et al. Positive regulatory domain I (PRDM1) and IRF8/PU.1 counter-regulate MHC class II transactivator (CIITA) expression during dendritic cell maturation. *J Biol Chem.* (2011) 286:7893–904. doi: 10.1074/jbc.M110.165431
13. Piskurich JF, Lin KI, Lin Y, Wang Y, Ting JPY, Calame K. BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nat Immunol.* (2000) 1:526–32. doi: 10.1038/82788
14. Kim SJ, Schatzle S, Ahmed SS, Haap W, Jang SH, Gregersen PK, et al. Increased cathepsin S in Prdm1(-/-) dendritic cells alters the TFH cell repertoire and contributes to lupus. *Nat Immunol.* (2017) 18:1016–24. doi: 10.1038/ni.3793
15. Goudot C, Coillard A, Villani AC, Gueguen P, Cros A, Sarkizova S, et al. Aryl hydrocarbon receptor controls monocyte differentiation into dendritic cells versus macrophages. *Immunity.* (2017) 47:582. doi: 10.1016/j.immuni.2017.08.016
16. Yu J, Angelin-Duclos C, Greenwood J, Liao J, Calame K. Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Mol Cell Biol.* (2000) 20:2592–603. doi: 10.1128/MCB.20.7.2592-2603.2000
17. Su ST, Ying HY, Chiu YK, Lin FR, Chen MY, Lin KI. Involvement of histone demethylase LSD1 in blimp-1-mediated gene repression during plasma cell differentiation. *Mol Cell Biol.* (2009) 29:1421–31. doi: 10.1128/MCB.01158-08
18. Ancelin K, Lange UC, Hajkova P, Schneider R, Bannister AJ, Kouzarides T, et al. Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat Cell Biol.* (2006) 8:623–30. doi: 10.1038/ncb1413
19. Ren B, Chee KJ, Kim TH, Maniatis T. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the groucho family of proteins. *Genes Dev.* (1999) 13:125–37. doi: 10.1101/gad.13.1.125
20. Roberts NA, Adams BD, McCarthy NI, Tooze RM, Parnell SM, Anderson G, et al. Prdm1 regulates thymic epithelial function to prevent autoimmunity. *J Immunol.* (2017) 199:1250–60. doi: 10.4049/jimmunol.1600941
21. Martins GA, Cimmino L, Shapiro-Shelef M, Szabolcs M, Herron A, Magnusdottir E, et al. Transcriptional repressor blimp-1 regulates T cell homeostasis and function. *Nat Immunol.* (2006) 7:457–65. doi: 10.1038/ni1320
22. Kallies A, Hawkins ED, Belz GT, Metcalf D, Hommel M, Corcoran LM, et al. Transcriptional repressor blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol.* (2006) 7:466–74. doi: 10.1038/ni1321
23. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, et al. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell.* (2010) 143:416–29. doi: 10.1016/j.cell.2010.09.039
24. Jang SH, Chen H, Gregersen PK, Diamond B, Kim SJ. Kruppel-like factor4 regulates PRDM1 expression through binding to an autoimmune risk allele. *JCI Insight.* (2017) 2:e89569. doi: 10.1172/jci.insight.89569
25. Melnikov SV, Manakongtreecheep K, Rivera KD, Makarenko A, Pappin DJ, Soll D. Muller's ratchet and ribosome degeneration in the obligate intracellular parasites microsporidia. *Int J Mol Sci.* (2018) 19:4125. doi: 10.20944/preprints201811.0508.v1

26. Minnich M, Tagoh H, Bonelt P, Axelsson E, Fischer M, Cebolla B, et al. Multifunctional role of the transcription factor blimp-1 in coordinating plasma cell differentiation. *Nat Immunol.* (2016) 17:331–43. doi: 10.1038/ni.3349
27. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gustafsdottir SM, et al. Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol.* (2002) 20:473–7. doi: 10.1038/nbt0502-473
28. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods.* (2006) 3:995–1000. doi: 10.1038/nmeth947
29. Kim SJ, Goldstein J, Dorso K, Merad M, Mayer L, Crawford JM, et al. Expression of blimp-1 in dendritic cells modulates the innate inflammatory response in dextran sodium sulfate-induced colitis. *Mol Med.* (2015) 20:707–19. doi: 10.2119/molmed.2014.00231
30. Park Y, Lee JM, Hwang MY, Son GH, Geum D. Nono binds to the CpG island of oct4 promoter and functions as a transcriptional activator of oct4 gene expression. *Mol Cells.* (2013) 35:61–9. doi: 10.1007/s10059-013-2273-1
31. Yadav SP, Hao H, Yang H-J, Kautzmann M-AI, Brooks M, Nellissery J, et al. The transcription-splicing protein NonO/p54^{nrb} and three NonO-interacting proteins bind to distal enhancer region and augment rhodopsin expression. *Hum Mol Genet.* (2014) 23:2132–44. doi: 10.1093/hmg/ddt609
32. Zhang C, Zhang MX, Shen YH, Burks JK, Zhang Y, Wang J, et al. TNF-alpha suppresses prolyl-4-hydroxylase alpha 1 expression via the ASK1-JNK-NonO pathway. *Arterioscl Throm Vas.* (2007) 27:1760–67. doi: 10.1161/ATVBAHA.107.144881
33. Lord CA, Savitsky D, Sitcheran R, Calame K, Wright JR, Ting JPY, et al. Blimp-1/PRDM1 mediates transcriptional suppression of the NLR Gene NLRP12/Monarch-1. *J Immunol.* (2009) 182:2948–58. doi: 10.4049/jimmunol.0801692
34. Hatzi K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. *J Exp Med.* (2015) 212:539–53. doi: 10.1084/jem.20141380
35. Chan YH, Chiang MF, Tsai YC, Su ST, Chen MH, Hou MS, et al. Absence of the transcriptional repressor blimp-1 in hematopoietic lineages reveals its role in dendritic cell homeostatic development and function. *J Immunol.* (2009) 183:7039–46. doi: 10.4049/jimmunol.0901543
36. Chang DH, Angelin-Duclos C, Calame K. BLIMP-1: trigger for differentiation of myeloid lineage. *Nat Immunol.* (2000) 1:169–76. doi: 10.1038/77861
37. Yang R, Masters AR, Fortner KA, Champagne DP, Yanguas-Casas N, Silberger DJ, et al. IL-6 promotes the differentiation of a subset of naive CD8(+) T cells into IL-21-producing B helper CD8(+) T cells. *J Exp Med.* (2016) 213:2281–91. doi: 10.1084/jem.20160417
38. Diehl SA, Schmidlin H, Nagasawa M, Blom B, Spits H. IL-6 triggers IL-21 production by human CD4(+) T cells to drive STAT3-dependent plasma cell differentiation in B cells. *Immunol Cell Biol.* (2012) 90:802–11. doi: 10.1038/icb.2012.17
39. Nish SA, Schenten D, Wunderlich FT, Pope S, Gao Y, Hoshi N, et al. T cell-intrinsic role of IL-6 signaling in primary and memory responses. *Elife.* (2014) 3:e01949. doi: 10.7554/eLife.01949
40. Li W, Karwacki-Neisius V, Ma C, Tan L, Shi Y, Wu F, et al. Nono deficiency compromises TET1 chromatin association and impedes neuronal differentiation of mouse embryonic stem cells. *Nucleic Acids Res.* (2020) 48:4827–38. doi: 10.1093/nar/gkaa213
41. Hallier M, Tavitian A, Moreau-Gachelin F. The transcription factor Spi-1/PU.1 binds RNA and interferes with the RNA-binding protein p54nrb. *J Biol Chem.* (1996) 271:11177–81. doi: 10.1074/jbc.271.19.11177
42. Carotta S, Willis SN, Hasbold J, Inouye M, Pang SH, Emslie D, et al. The transcription factors IRF8 and PU.1 negatively regulate plasma cell differentiation. *J Exp Med.* (2014) 211:2169–81. doi: 10.1084/jem.20140425
43. Nagy M, Chapuis B, Matthes T. Expression of transcription factors Pu.1, Spi-B, Blimp-1, BSAP and oct-2 in normal human plasma cells and in multiple myeloma cells. *Br J Haematol.* (2002) 116:429–35. doi: 10.1046/j.1365-2141.2002.03271.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 11 August 2020
doi: 10.3389/fimmu.2020.01897

Dietary Glucose Consumption Promotes RALDH Activity in Small Intestinal CD103$^+$CD11b$^+$ Dendritic Cells

Hyun-Ja Ko[1†‡], Sung-Wook Hong[1†‡], Ravi Verma[2,3], Jisun Jung[2], Minji Lee[2], Nahyun Kim[2], Daeun Kim[2], Charles D. Surh[1,2§], Kwang Soon Kim[2], Dipayan Rudra[2] and Sin-Hyeog Im[2,3*]*

[1] Academy of Immunology and Microbiology, Institute for Basic Science, Pohang-si, South Korea, [2] Division of Integrative Biosciences and Biotechnology, Department of Life Sciences, Pohang University of Science and Technology, Pohang-si, South Korea, [3] ImmunoBiome Inc., Pohang-si, South Korea

Retinal dehydrogenase (RALDH) enzymatic activities catalyze the conversion of vitamin A to its metabolite Retinoic acid (RA) in intestinal dendritic cells (DCs) and promote immunological tolerance. However, precise understanding of the exogenous factors that act as initial trigger of RALDH activity in these cells is still evolving. By using germ-free (GF) mice raised on an antigen free (AF) elemental diet, we find that certain components in diet are critically required to establish optimal RALDH expression and activity, most prominently in small intestinal CD103$^+$CD11b$^+$ DCs (siLP-DCs) right from the beginning of their lives. Surprisingly, systematic screens using modified diets devoid of individual dietary components indicate that proteins, starch and minerals are dispensable for this activity. On the other hand, in depth comparison between subtle differences in dietary composition among different dietary regimes reveal that adequate glucose concentration in diet is a critical determinant for establishing RALDH activity specifically in siLP-DCs. Consequently, pre-treatment of siLP-DCs, and not mesenteric lymph node derived MLNDCs with glucose, results in significant enhancement in the *in vitro* generation of induced Regulatory T (iTreg) cells. Our findings reveal previously underappreciated role of dietary glucose concentration in establishing regulatory properties in intestinal DCs, thereby extending a potential therapeutic module against intestinal inflammation.

Keywords: retinal dehydrogenase (RALDH), regulatory T cells (Treg), dendritic cells (DCs), LP-DCs, retinoic acid (RA), vitamin A, immune regulation, dietary glucose

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Bart Everts,
Leiden University Medical Center, Netherlands
Maria Rosa Bono,
University of Chile, Chile
Andrew John Stagg,
Queen Mary University of London, United Kingdom

***Correspondence:**
Dipayan Rudra
dipayanrudra@gmail.com;
rudrad@postech.ac.kr
Sin-Hyeog Im
iimsh@postech.ac.kr

†Present address:
Hyun-Ja Ko,
KoBioLabs Inc., Seoul, South Korea
Sung-Wook Hong,
Department of Microbiology and Immunology, Centre for Immunology, University of Minnesota Medical School, Minneapolis, MN, United States

‡*These authors have contributed equally to this work*

§*Deceased*

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *28 April 2020*
Accepted: *14 July 2020*
Published: *11 August 2020*

Citation:
Ko H-J, Hong S-W, Verma R, Jung J, Lee M, Kim N, Kim D, Surh CD, Kim KS, Rudra D and Im S-H (2020) Dietary Glucose Consumption Promotes RALDH Activity in Small Intestinal CD103$^+$CD11b$^+$ Dendritic Cells. Front. Immunol. 11:1897. doi: 10.3389/fimmu.2020.01897

INTRODUCTION

Intestinal dendritic cells (DCs) are critical for the initiation and regulation of innate and adaptive immunity by delivering self or foreign antigens to T cells (1–3). The intestine is spontaneously exposed to innumerable antigens comprising of intestinal microbes (4, 5) as well as dietary components (6, 7). To maintain immune homeostasis, intestinal DCs regulate the balance between the tolerogenic immune response by inducing CD4$^+$Foxp3$^+$ regulatory T cells (Treg cells) (8–10) and the protective immune responses by inducing effector T cells (11, 12). Dysregulation of this balance by harmful pathogens or dietary intake results in inflammatory disorders (13, 14), such as inflammatory bowel disease (IBD) (15, 16), celiac disease (17) and food allergy (18).

Intestinal DCs are located in the Peyer's Patches (PPs), mesenteric lymph nodes (MLNs) and lamina propria (LP) and comprise cellular subsets that have different origins and functions (1, 2, 19–23). Among these DC subtypes, intestinal $CD103^+$ DCs have the unique function that metabolizes vitamin A to retinoic acid (RA) through the activation of aldehyde dehydrogenase 1, member A2 [Aldh1a2, also called retinaldehyde dehydrogenase (RALDH2)] enzyme (24, 25). The RA produced by intestinal DCs play an important role in orchestrating immune responses; imprinting gut-homing specificity on T cells, B cells and innate lymphoid cells (ILCs), inducing IgA-producing B cells, promoting TGF-β-dependent differentiation of induced Treg cells, suppressing the differentiation of Th17 cells, enhancing IL-22 production by γδ T cells and ILCs, as well as inducing effector functions in T cells (26–39).

While vitamin A derived from dietary intake can induce RALDH enzymatic activity, the RA produced from intestinal epithelial cells (IECs) by RALDH1 and stroma cells in LP and MLN by RALDH2, in a trans activating mechanism is also capable of inducing RALDH expression in intestinal DCs (40–44). Furthermore, recent data suggest that RA is also involved in the development of a gut homing precursor for intestinal DCs in the bone marrow as well as is required for their transcriptional programming and maturation (45, 46). Several endogenous factors that regulate RALDH expression in LP-DCs are also reported. Cytokines such as IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) induce or enhance the expression of RALDH enzymes in LP-DCs, while prostaglandin E2 (PGE2) negatively regulates RALDH activity through the induction of inducible cyclic AMP early repressor (ICER) (24, 47–49). Despite these findings, whether additional components in diet can induce RALDH activity in the intestine and promote immune tolerance, remains unknown. In this study, we uncover a hitherto unknown role of dietary glucose in shaping up intestinal immunological tolerance by facilitating RALDH expression specifically in intestinal LP-DCs.

RESULTS

Mice Administered Antigen Free Diet Have Defects in Development and RALDH Activity in $CD103^+CD11b^+$ siLP-DCs

To investigate the influence of commensal microbiota and food components on intestinal immunity, we utilized the previously established "Antigen free (AF)" mice model, where germ-free (GF) mice are raised on well-defined elemental diet [termed "Antigen free diet" (AFD)] devoid of macromolecules such as proteins and starches (50). When DCs in small intestine were assessed, we observed comparable frequencies of $CD11c^+MHC\text{-}II^+$ siLP-DCs in specific pathogen free (SPF), GF and AF mice (**Figures 1A,B**). However, in-depth analyses revealed alteration in the frequencies of tolerogenic DC subtypes. The frequency of $CD103^+CD11b^+$ siLP-DCs, a subset known to be a major tolerogenic DC population, was slightly, but significantly lower in AF, when compared to SPF and GF mice (**Figure 1C**). A compensatory increase on the other hand was observed in the $CD103^+CD11b^-$ siLP-DC compartment. More interestingly, while the expression of the characteristic DC surface markers largely remained comparable in all three groups (**Figure S1A**), the expression of all three representative genes tested, namely *Aldh1a2*, Indoleamine-pyrrole 2,3-dioxygenase 1 (*Ido1*) and Transforming growth factor beta 1 (*Tgfb1*) that are functionally implicated in tolerogenic phenotype of $CD103^+CD11b^+$ DCs, were dramatically reduced in AF mice (**Figure 1D**). Interestingly, the expression of *Aldh1a2* was found to be specifically reduced in mice raised under AFD, a phenomenon that was not observed in GF mice (**Figure 1D**, left panel). On the other hand, the absence of gut microbiota appeared to partially influence the expression of *Ido1* and *Tgfb1*, which was further enhanced by AFD (**Figure 1D**, middle and right panel). These results indicated that certain dietary components, otherwise absent or under-represented in AFD have most specific and the largest influence on the expression of *Aldh1a2*. For this study, we therefore focused on the influence of normal diet on RALDH activity in siLP-DCs.

RALDH is an enzyme that irreversibly metabolizes vitamin A to RA, which in turn acts as a key modulator of mucosal immune responses (38, 51–53). To determine whether in concert to its reduced expression, the function of RALDH in LP-DCs from AF mice was also negatively affected, we next examined RALDH enzyme activity in LP-DCs from SPF, GF and AF mice using the ALDEFLUOR assay. In this assay, which has been previously employed in the context of $CD103^+$ LP-DCs and MLN-DCs (43, 44), the RALDH enzyme activity is measured in individual cells by flow cytometry with a fluorescent substrate based assay system (54, 55). In agreement with the results obtained by real-time PCR analysis, $CD103^+$ siLP-DCs (both $CD11b^+$ and $CD11b^-$ subsets) from AF mice displayed significantly lower enzyme activity when compared with SPF and GF mice (**Figures 1E,F**, **Figure S1B**). Of note, the characteristic frequencies of the aforementioned siLP-DC subtypes in SPF, GF and AF mice remained unaltered even after performing this assay, suggesting that this enzyme assay did not interfere with the phenotype of siLP-DCs (**Figure S1C**).

In order to further understand the role of dietary components on RALDH activity, we next analyzed siLP-DC RALDH activity in mice at different stages of their lives, after subjecting them to specific dietary conditions. We observed that RALDH activity in pre-weaned GF mice (2-3 weeks of age) was significantly lower than in adult GF mice, and comparable to AF mice. This was dramatically restored to the level equivalent to adult GF mice within a week after weaning (**Figure 2A**). Furthermore, when mice raised in AF condition were fed with Normal Chow Diet (NCD), RALDH activity in $CD103^+CD11b^+$ siLP-DCs was promptly recovered within a week (**Figure 2B**). Mirroring this, an opposite phenomenon was observed when NCD in GF mice was replaced with AFD (**Figure 2C**). These results suggested that dietary component(s) in NCD, absent in AFD, is required as the initial trigger for RALDH gene expression after mice are weaned, thereby promoting enzyme activity as well as homeostasis of $CD103^+CD11b^+$ siLP-DCs.

The above results also implied that supplementing AF mice with RA, the final product of the enzymatic reaction and a known feedback inducer of RALDH activity (40), would be sufficient in driving RALDH activity in these mice. Indeed, when adult AF

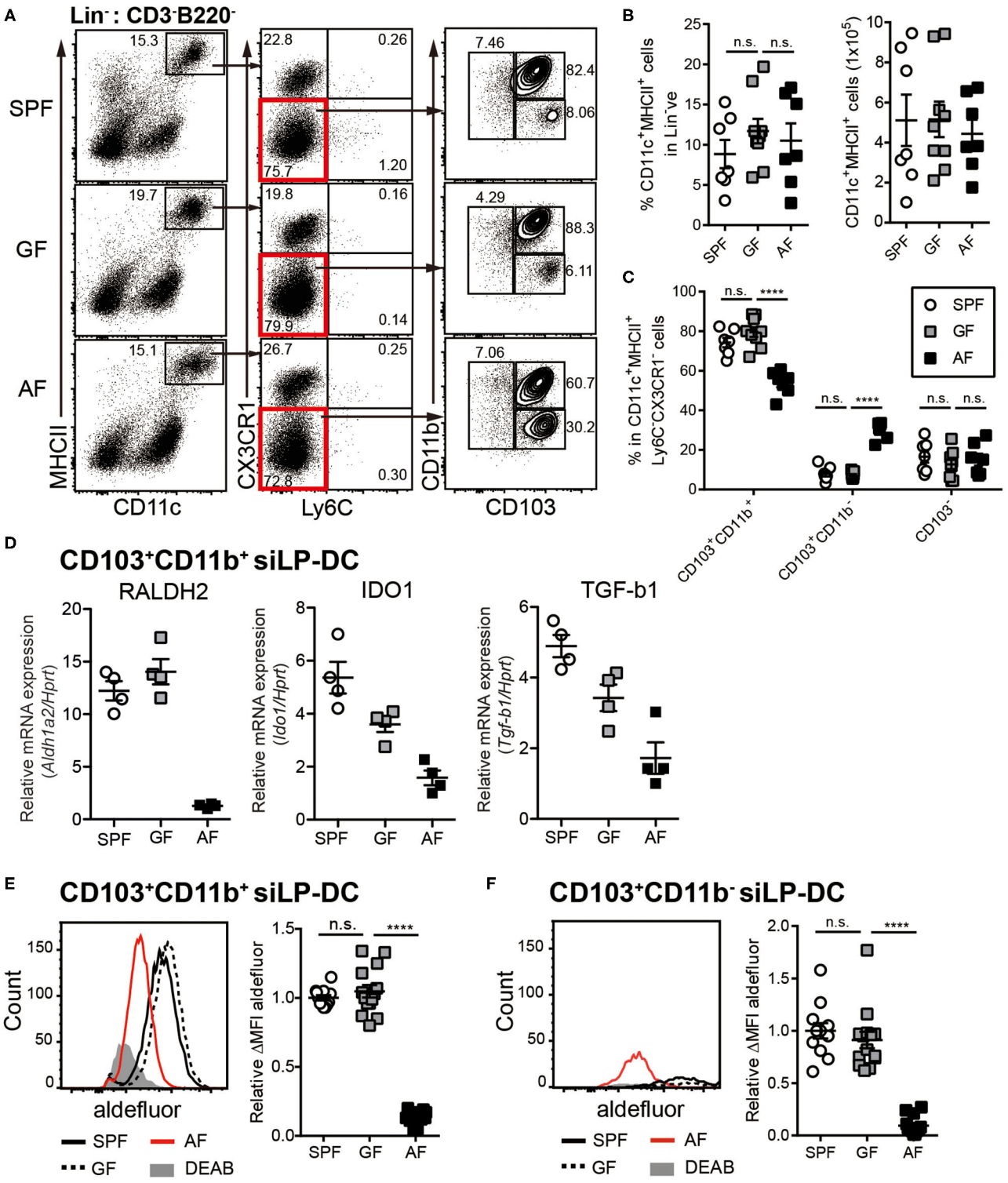


FIGURE 1 | Dietary intake affects differentiation and RALDH activity in CD103⁺ siLP-DCs. Cell suspensions were prepared from siLP harvested from age-matched adult (6∼12-week-old) SPF, GF and AF mice and phenotypic and RALDH activity analyses of siLP-DCs were carried out. **(A)** Representative fluorescence-activated

(Continued)

FIGURE 1 | cell sorting (FACS) plots of siLP-DC subpopulations gated on Lin$^-$ (CD3$^-$B220$^-$) cells. **(B)** Statistical quantification of percentage (left) and total numbers (right) of CD11c$^+$MHC-II$^+$ cells in mice from indicated experimental groups. **(C)** Graph displays percentage of siLP-DC subpopulations in CD11c$^+$MHC-II$^+$ cells. **(B,C)** Data are combined from four independent experiments. **(D)** Real-time analyses of mRNA expression of indicated gene products normalized against *Hprt* mRNA levels. **(E,F)** Representative FACS plots (left) and quantification (right) of relative mean fluorescence intensity (MFI) of aldefluor in CD103$^+$CD11b$^+$ **(E)** and CD103$^+$CD11b$^-$ **(F)** siLP-DC subpopulations from indicated groups. DEAB is a RALDH inhibitor. ΔMFI is calculated by subtracting background (DEAB) MFI from aldefluor MFI. Relative ΔMFI indicates ratio of ΔMFI in experimental samples vs. control. Data are combined from six independent experiments. MEAN ± SEM are indicated. Statistical significance was determined by one-way ANOVA **(B,E,F)** and two-way ANOVA **(C)** with Turkey's multiple comparison tests. ****$p < 0.0001$, *n.s.*, not statistically significant.

mice were supplemented in their diet with RA, it resulted in complete recovery of RALDH activity (**Figure 2D**). Interestingly in all the cases, changes in RALDH activity also correlated with the frequencies of CD103$^+$CD11b$^+$ siLP-DCs, suggesting its role in differentiation as well as function of these cells. Of note, while the above results were obtained in a GF setting, the basic findings from these experiments could also be recapitulated in mice raised in SPF conditions, thereby confirming that mice with normal repertoire of gut flora are equally affected by dietary components with respect to RALDH activity in siLP-DCs (**Figures S2A–C**).

RALDH Activities in Different Intestinal RA-Producing Cells Are Differentially Affected by Diet

We next sorted to understand whether the influence of diet on RALDH activity is an LP-DC specific phenomenon, or whether other RALDH expressing cells are also affected. It is well-established that within the gut associated lymphoid tissues, besides LP-DCs, RA converting enzymes are also expressed in LP associated stroma cells (LP-SCs), small intestine epithelial cells (IECs), as well as MLN-DCs. The RA produced from these cells, in particular IECs, is known as a local source of RA for inducing the RALDH expression in CD103$^+$ siLP-DCs (40–44).

The non-hematopoietic SCs in secondary lymph nodes comprise three different cell types based on the expression of surface markers. Lymphatic stroma cells [LSCs, also called fibroblast reticular cells (FRC)], lymphatic endothelial cells (LECs) and blood endothelial cells (BECs) (56, 57). Among LP-SCs (CD45$^-$EpCAM$^-$) in small intestine, the LSCs that expressed podoplanin (Pdpn) and are CD31$^-$, were found to be capable of activating RALDH enzymes (**Figure 3A**), as previously reported (43). Interestingly, unlike LP-DCs, the RALDH activity in LP-SCs remained comparable between GF and AF mice (**Figure 3B**). In contrast, when IECs were analyzed, the expression of *Aldh1a1* (RALDH1), the major gene encoding for RALDH enzyme in these cells, was found to be reduced in AF mice (**Figure 3C**). Of note, while the IECs are well-established to have RALDH activity (27, 58–61), the baseline of this activity in these cells is known to be significantly lower than LP-DCs (62, 63). Therefore, our attempt to measure RALDH activity in IECs was unsuccessful due to lower sensitivity of the ALDEFLUOR assay. However, albeit comparatively lower RALDH activity on a per cell basis, the cumulative contribution of IECs in RA production, is understandably of larger significance since numerically there are many more IECs than the other cell types in the intestine.

Finally, when MLN-DCs were analyzed, the RALDH activity in particular within the CD103$^+$CD11b$^+$ DC population in AF mice was found to be significantly, albeit to a lesser extent, lower than that of GF mice (**Figure 3D**). Taken together these results suggested that dietary components differentially influence RALDH activity in different regulatory DC populations. Whereas, MLN-DCs and IEC are affected, LP-SCs appeared to remain unaffected from dietary contributions. These results also implied that the overall reduction of RA synthesis cumulatively among these cell types eventually contributed to the reduced RALDH activity in the LP-DCs in AF mice.

Proteins, Starches, and Minerals in Diet Do Not Influence RALDH Activity in CD103$^+$CD11b$^+$ siLP-DCs

Since for this study we focused on RALDH activity in siLP-DCs, we next wished to define which dietary factors were required to trigger the initial RALDH activity in these cells. While in our initial findings we observed both the subtypes CD103$^+$CD11b$^+$ and CD103$^+$CD11b$^-$ siLP-DCs to have reduced RALDH activity in AF mice (**Figures 1E,F**), the cell recovery of CD103$^+$CD11b$^-$ siLP-DCs from SPF and GF mice were low and the level of enzyme activity in this cell type showed variability among individual mice (**Figures 1C,F**). Therefore, henceforth in this study, we focused on the RALDH activity in CD103$^+$CD11b$^+$ siLP-DCs.

We first confirmed that the reduction in RALDH activity in CD103$^+$CD11b$^+$ siLP-DCs was not a consequence of low Vitamin A content in AF diet, thereby compromising the precursor for the assayed reaction. Based on information of food compositions from the suppliers and from our pervious report, final consumption of vitamin A per day by GF and AF mice were comparable [**Table S1** and (50)]. Nonetheless, there remained a possibility that albeit equal consumption, the absorption of vitamin A into the small intestine may be compromised in AF mice. However, when GF mice were weaned on AF diet supplemented with 10 times more vitamin A in the usual form of "oil mix" or were administrated additional "oil mix" by oral gavage, failed to recover the reduction in RALDH activity in CD103$^+$CD11b$^+$ siLP-DCs (**Figure S3**). We thus concluded that mere unavailability of the precursor vitamin A was not a cause of reduced RALDH activity in these cells.

To this end, we modified the compositions of purified diet by removing individual food components (**Table S2**), with

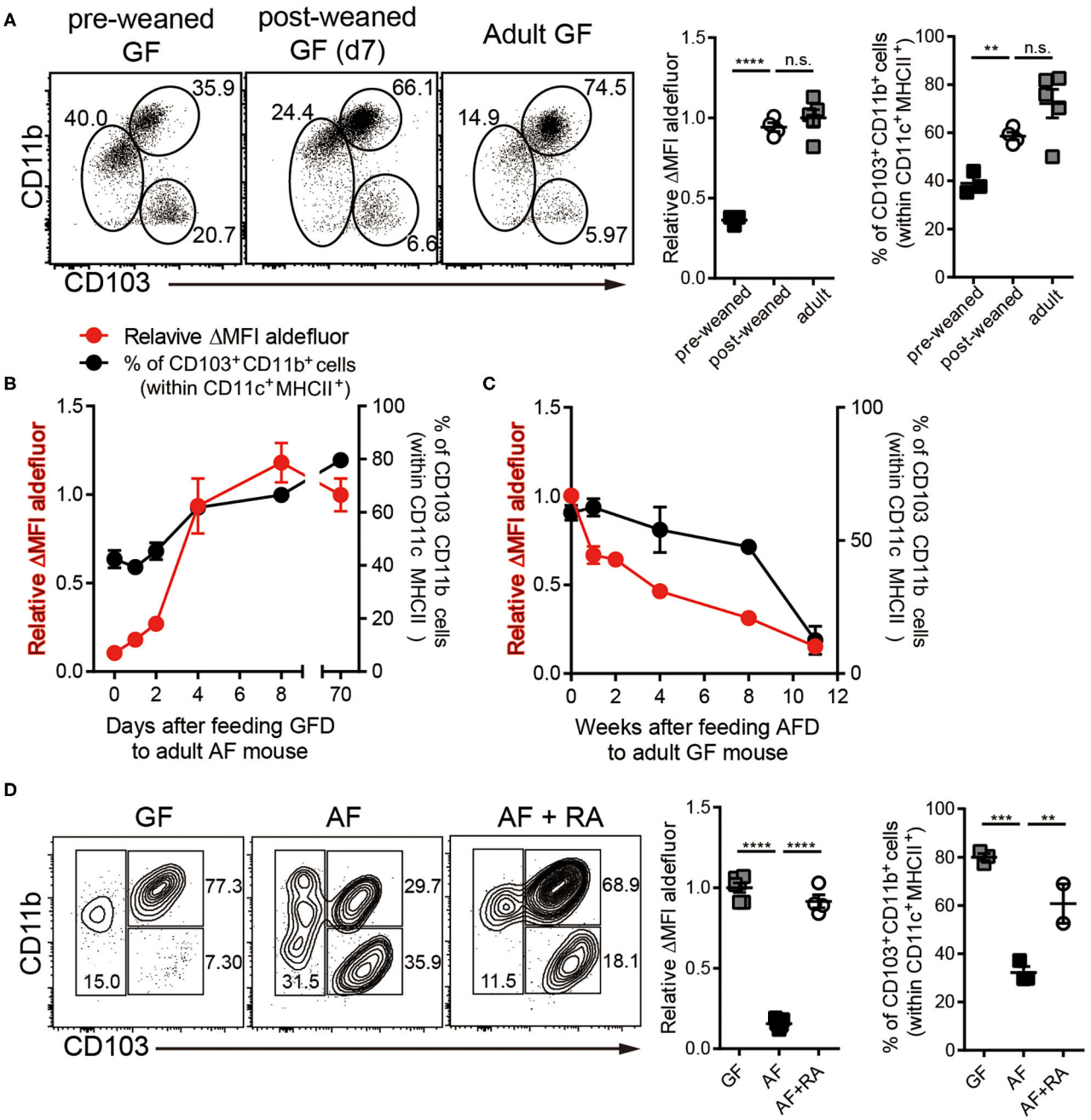


FIGURE 2 | Dietary components in normal chow readily trigger and maintain RALDH activity in siLP-DCs in mice after weaning. Cell suspensions prepared from siLP were subjected to ALDEFLUOR assays and RALDH activity in $CD103^+CD11b^+$ siLP-DCs and percentage of siLP-DC subpopulations in $CD11c^+MHC\text{-}II^+$ cells were analyzed by flow cytometry. **(A)** GF mice (3-weeks old) before weaning (pre-weaned), GF mice weaned onto NCD for 7 days and adult GF mice were analyzed. **(B)** RALDH activity and frequencies of siLP-DC subpopulations in $CD11c^+MHC\text{-}II^+$ cells in adult AF mice (8∼12-week-old) after feeding NCD for 0, 1, 2, 4, 8, and 70+ days. **(C)** RALDH activity and frequencies of siLP-DC subpopulations in $CD11c^+MHC\text{-}II^+$ cells in adult GF mice (8∼12-week-old) after feeding AFD for 0, 1, 2, 4, 8, and 11+ weeks. **(D)** RALDH activity and frequencies of siLP-DC subpopulations in $CD11c^+MHC\text{-}II^+$ cells in adult GF, AF, or AF mice that were administered intra peritoneal injection of all-trans RA (500 μg per mouse in soybean oil) every other day for 7 days. Data are combined from two to three independent experiments. MEAN ± SEM are indicated. **(B,C)** Statistical significance was determined by one-way ANOVA with Turkey's multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, *n.s.*, not statistically significant.

the presumption that taking out or adding back individual components in otherwise well-defined diet may lead us toward identifying the dietary component required to trigger RALDH activity. To obtain relatively accurate results under *in vivo* settings, the modified diets were designed to contain similar amount of vitamin A as in NCD (**Tables S1, S2**) and the

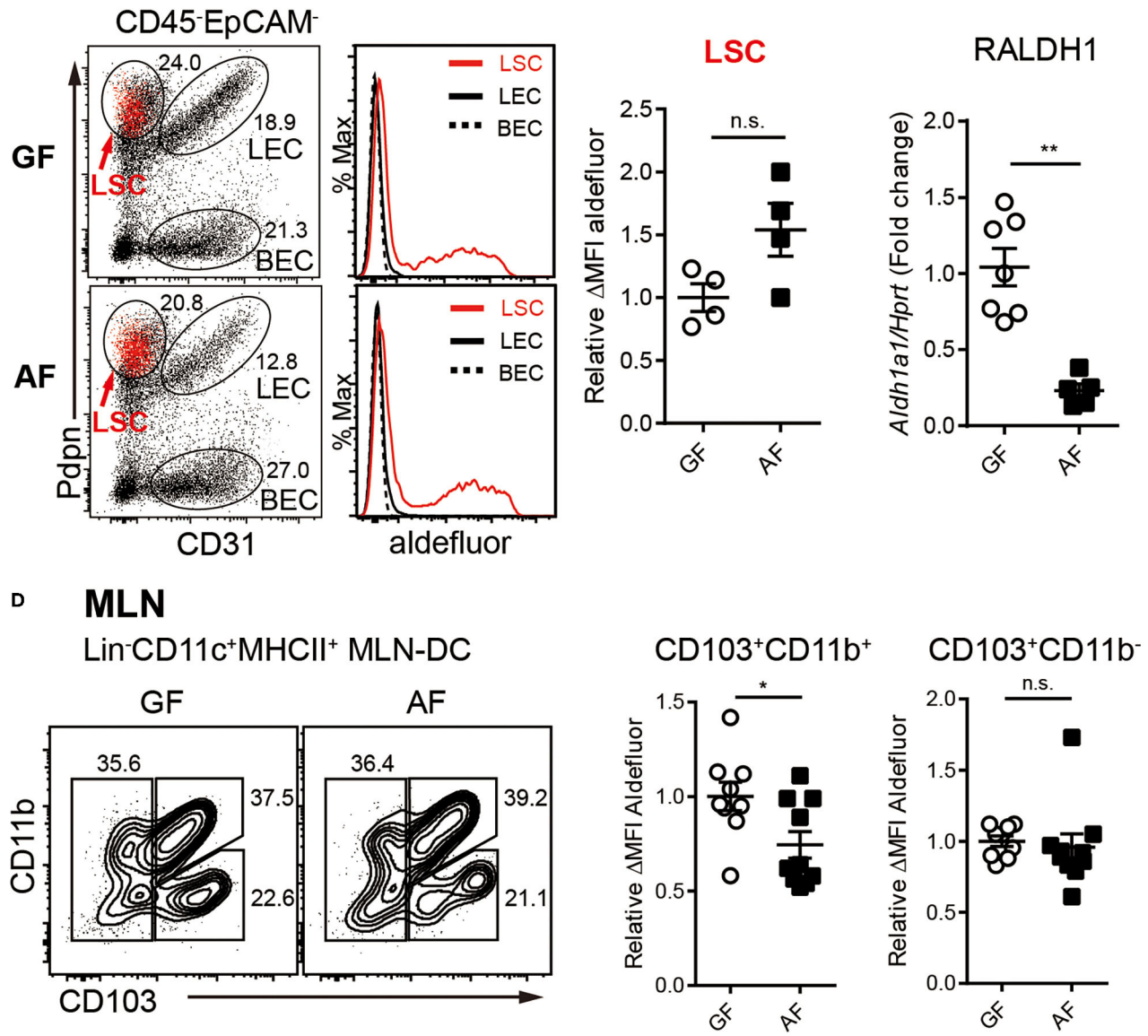


FIGURE 3 | RALDH activity in intestinal RA-producing cells is differentially regulated by diet. **(A–C)** Cell suspensions from MLN and siLP harvested from age-matched adult (6∼12-week-old) GF and AF mice were subjected to ALDEFLUOR assays and RALDH activity of LP-SCs and MLN-DCs were analyzed by flow cytometry. **(A)** Representative FACS plot of LP-SC distinguished by CD31 and Pdpn from CD45-EpCAM- cells. Red dots in FACS plot indicate aldefluor positive cells. Histograms depict the MFI of aldefluor in LP-SC subtypes. **(B)** Graph displays the level of RALDH activity in LSCs. Data is combined from two independent experiments. **(C)** IECs were isolated from small intestine (SI) by stripping with EDTA and analyzed for expression of *Aldh1a1* (RALDH1) by real-time PCR, upon normalization to *Hprt* mRNA levels. Fold change indicates ratio target gene in experiment/control. Data are combined from four independent experiments. **(D)** Representative FACS plots of MLN-DC subpopulations distinguished by CD103 and CD11b from $CD11c^{+}MHC\text{-}II^{+}Lin^{-}$ cells and the RALDH activity in $CD103^{+}CD11b^{+}$ MLN-DCs and $CD103^{+}CD11b^{-}$ MLN-DCs. Data are combined from four independent experiments. MEAN ± SEM are indicated. Statistical significance was determined by two-tailed unpaired t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, *n.s.*, not statistically significant.

experiments were performed primarily in GF condition in order to eliminate the influence of microbiota. In addition, to avoid any influence from NCD during the pre-weaned period, neonate AF mice were utilized and these mice were weaned onto each modified diet for 3–4 weeks.

As a starting point, we took advantage of two commercially available diets with well-defined dietary compositions. In the so-called amino acid defined diet (AAD*), like the AFD employed so far, proteins were replaced with amino acids. However, there were several differences between their compositions (**Table S2**). While

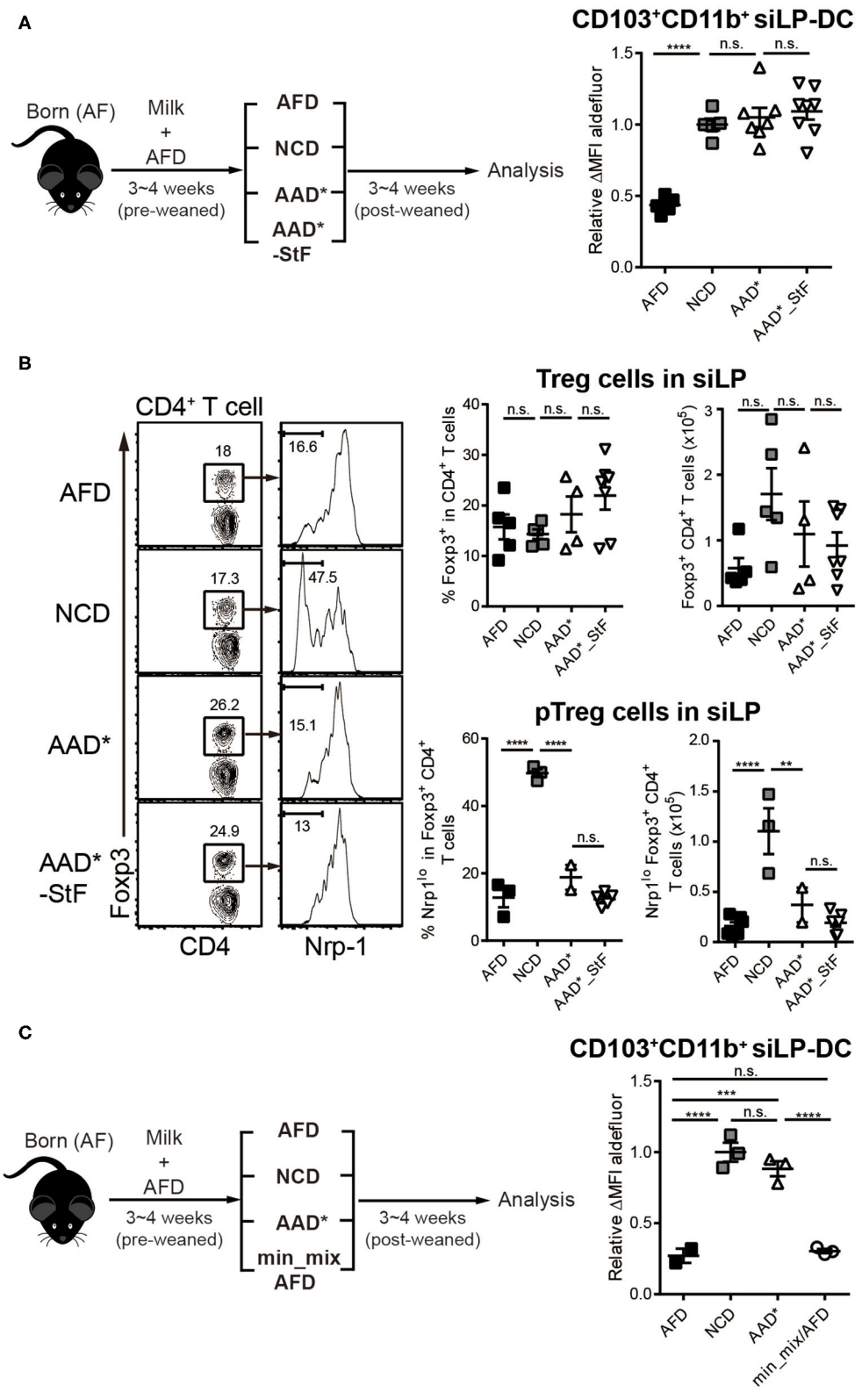


FIGURE 4 | Depletion of macromolecules from purified diet do not alter the RALDH activity in CD103$^+$CD11b$^+$ siLP-DCs. AF mice (3∼4-week-old) were weaned onto specific diets for 3∼4 weeks following which the indicated analyses were carried on. **(A)** A cartoon depicting experimental scheme (left panel). AAD* is a sterilized form

(Continued)

FIGURE 4 | of Amino acid-defined diet (AAD) that contains three times more vitamin A than AAD, where protein macromolecules are replaced with amino acids. AAD*_StF indicates AAD* from which cornstarch and maltodextrin are removed. The level of RALDH activity in $CD103^+CD11b^+$ siLP-DCs in the indicated groups are presented by relative aldefluor ΔMFI (right panel). **(B)** Representative FACS plots (left panel), frequencies (middle panel) and absolute numbers (right panel) of total $CD4^+Foxp3^+$Treg cell and $CD4^+Foxp3^+Nrp1^{lo}$ peripheral Treg (pTreg) cell populations in siLP of mice subjected to the indicated diet regimes. **(C)** Experimental scheme (left panel), and relative aldefluor ΔMFI of siLP-DCs in the indicated experimental groups (right panel). Min_mix AFD indicates AFD mixing with the mineral mix powder (TD.94049). Data are combined from four independent experiments. MEAN ± SEM are indicated. Statistical significance was determined by one-way ANOVA with Turkey's multiple comparison test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, *n.s.*, not statistically significant.

AFD is a liquid diet where the fibers are provided as cellulose bedding, AAD* diet are edible solid pellets with cellulose mixed with the food components. Unlike AFD, AAD* diet contained starches in the form of maltodextrin and corn starch, and while the source of sugar in AFD was 22% glucose, that in AAD* was 37.1% sucrose. Furthermore, in terms of mineral composition, there are substantial differences between the groups. The second commercially available diet is AAD*_StF, which was largely similar to AAD*, but was devoid of starches. Note, the "*" in AAD* indicates a sterilizable form of the diet which is otherwise similar to its traditional form (AAD), but with three times more vitamin A to account for presumed losses during sterilization by irradiation. Surprisingly, the mice groups weaned in both AAD* and AAD*_StF showed a complete recovery of RALDH activity in $CD103^+CD11b^+$ siLP-DCs, to an extent similar to the control NCD fed group (**Figure 4A**). These results were independent of microbiota, since the characteristic drop in RALDH activity of $CD103^+CD11b^+$ siLP-DCs in mice raised in SPF conditions could also be recovered by AAD (**Figure S2D**). Taken together these findings led to two important conclusions. First, starches are not involved. Second, antigens in the form of peptides, derived from proteins are also dispensable as far as RALDH activity in $CD103^+CD11b^+$ siLP-DCs is concerned. To this end, we also considered a possibility that an artifact arising from unaccounted protein contamination in the amino acid defined diets may be responsible for the observed recovery of RALDH activity. We therefore quantified the generation of peripheral Regulatory T (pTreg; also referred to as iTreg when induced *in vitro*) cell population in the siLP of these mice. pTreg cells are a type of Treg cells that are extrathymically generated primarily at mucosal sites, and are distinguished from their thymic tTreg counterparts by the lack of expression of the membrane bound co-receptor neuropilin-1 (Nrp1) (64). Notably, in a previous report we have demonstrated that diet derived proteins are the primary cause for the generation of $CD4^+Foxp3^+Nrp1^-$ pTreg cells in the small intestine (50), and AF mice display dramatically reduced pTreg population in siLP. Indeed we found that while total $Foxp3^+$ Treg populations, comprising of tTreg and pTreg cells, remained comparable, the frequencies and numbers of $Foxp3^+Nrp1^-$ pTreg cells among total Treg population could only be recovered in mice fed with NCD, and not AAD* and AAD*_StF (**Figure 4B**). Therefore, the recovery of RALDH activity in AAD* and AAD*_StF groups were not due to any protein contamination in the AAD* diet.

We next wished to exclude the possibility that differences in minor food components such as minerals, between AFD and purified diet (**Table S2**), was responsible for differences in RALDH activity. For that we prepared AFD by supplementing with mineral mix powder (TD.94049) derived from AAD* (min_mix/AFD). As shown in **Figure 4C**, min_mix/AFD failed to induce RALDH activity. Taken together, these results concluded that proteins, starches and minerals in diet were not responsible for the induction of RALDH activity in $CD103^+CD11b^+$ siLP-DCs.

Optimum Glucose Level in Diet Is Required To Induce RALDH Activity in $CD103^+CD11b^+$ siLP-DCs

If the macromolecules and micromolecules in diet were not involved, lack of which factors in AFD compromise RALDH activity in $CD103^+CD11b^+$ siLP-DCs? To investigate further, we compared the food compositions between AAD*_StF and AFD (**Table S2**), which were the closest among the four types of diets tested. In case of AAD*_StF, this diet does not contain proteins and starches as in AFD, but does contain unknown dietary factor(s) responsible for the induction of RALDH activity in siLP-DCs. There are few differences between the composition of these two diets; (1) the diet forms (pellet vs. liquid), (2) the carbohydrate sources (sucrose vs. glucose), (3) the amount of carbohydrate (sucrose ∼50% vs. glucose ∼22%). Based on this observation, as well as in order to minimize the differences in diet forms, we first generated a liquid form of AFD with 50% of sucrose (AFD_S500) or 50% glucose (AFD_G500). Germ free diet (GFD) or AAD*_StF were used as positive controls. AFD with usual 22% glucose, designated as AFD_G220 here, was used as negative control. The neonate AF mice were weaned onto each diet for 3–4 weeks and were analyzed for RALDH activity in $CD103^+CD11b^+$ siLP-DCs (**Figure 5A**, left panel). Indeed, AFD supplemented with 50% sucrose resulted in significantly enhanced RALDH activity in these cells. More interestingly, we observed a similar increase in RALDH activity in $CD103^+CD11b^+$ siLP-DCs even when the source of carbohydrate was changed to 50% glucose instead of sucrose (**Figure 5A**, middle panel). These effects were found to be specific for $CD103^+CD11b^+$ siLP-DCs, since RALDH1 expression in IECs remained unaltered upon carbohydrate supplementation (**Figure 5A**, right panel). These results suggested that regardless of the source of carbohydrate, its optimum concentration is important, and glucose being a monosaccharide unit of carbohydrate is sufficient for the initial triggering of RALDH activity in siLP-DCs.

In order to further understand whether the positive effect of dietary carbohydrate supplementation is specific for RALDH activity, or if it can also affect differentiation of other immune cells, we determined the frequencies of Th1, Th2, and pTreg

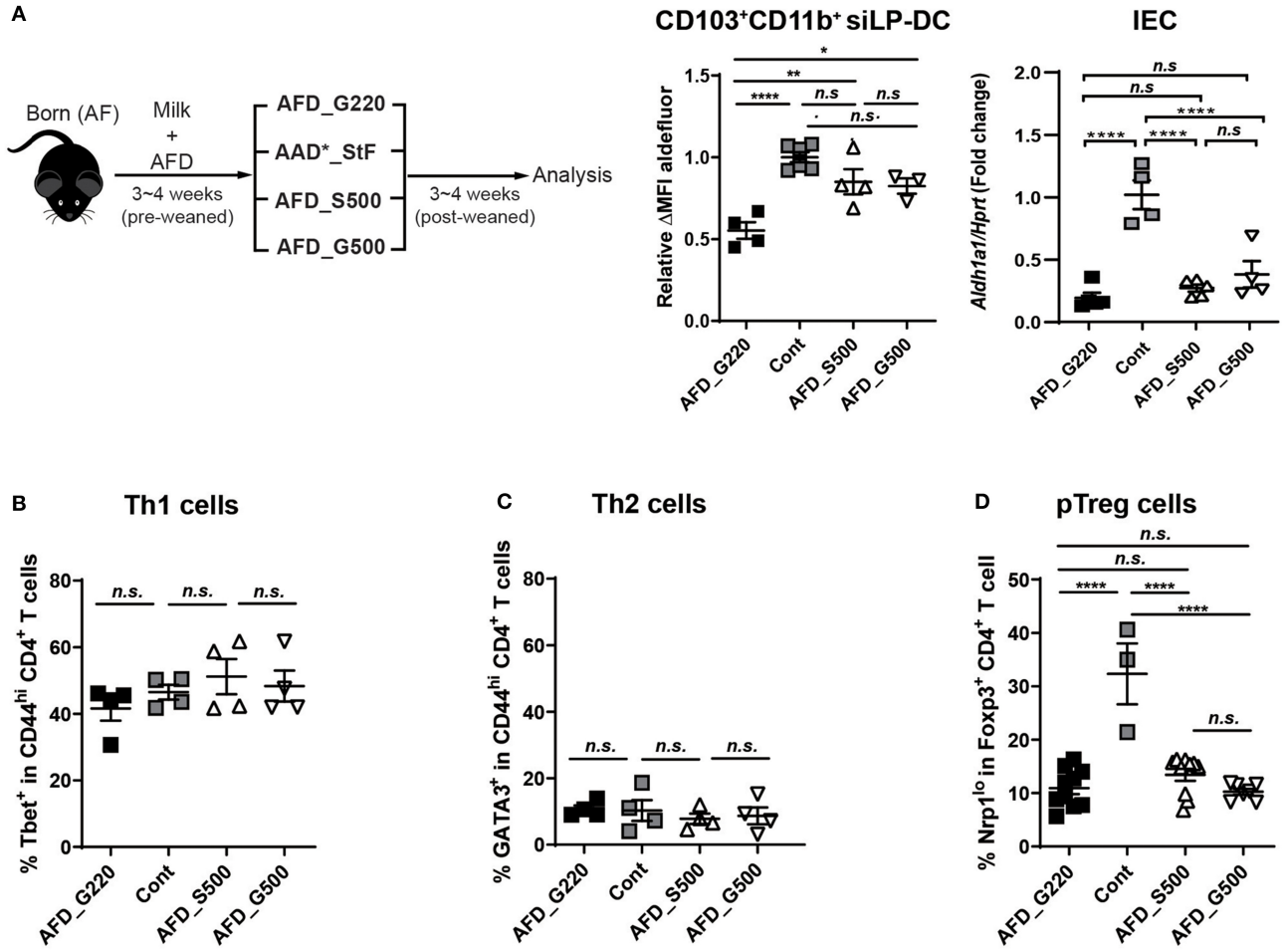


FIGURE 5 | Supplementing glucose in AF diet restores RALDH activity in CD103$^+$CD11b$^+$ siLP-DCs. **(A)** Cartoon depicting experimental scheme (left panel). Neonate AF mice were weaned onto specific diets for 3~4 weeks. AFD contains 22% of glucose (AFD_G220). AFD_G500 indicates 50% of glucose in AFD and AFD_S500 indicates 50% of sucrose in AFD. AAD*_StF was utilized as a positive control of RALDH activity in LP-DCs. ALDEFLUOR assays were performed on cell suspensions from siLP and RALDH activity of siLP-DC was analyzed by flow cytometry. The level of RALDH activity in CD103$^+$CD11b$^+$ siLP-DCs is represented as relative ΔMFI of aldefluor (middle panel). *Aldh1a1* expression was also determined in sorted IECs relative to *Hprt* control (right panel). Data are combined from two independent experiments. **(B–D)** Graphs display the percentage of Tbet$^+$ in CD4$^+$ T cells (Th1) **(B)**, GATA3$^+$ in CD4$^+$ T cells (Th2) **(C)**, and Nrp1^{lo} populations among in CD4$^+$Foxp3$^+$ Treg cells (pTreg) **(D)**. Data are combined from at least two independent experiments. MEAN ± SEM are indicated. Statistical significance was determined by one-way ANOVA with Turkey's multiple comparison test, $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, *n.s.*, not statistically significant.

cells in these mice. Supplementation of AFD with additional sucrose or glucose did not affect Tbet$^+$ Th1 or Gata3$^+$ Th2 cells (**Figures 5B,C**); neither recovered the characteristically reduced pTreg population in siLP (**Figure 5D**).

We next directly investigated the effect of glucose on RALDH activity by employing *in vitro* culture conditions. SPL-DCs, MLN-DCs, and CD11c$^+$ siLP-DCs were magnetically purified and cultured either without glucose (in commercially available glucose-free media), without glucose but in the presence of RA, with glucose, or in the presence of glucose and RA for 20 h, and then measured for RALDH activity. In terms of base-line RALDH activity, as expected, SPL-DCs displayed the least, which was significantly increased in the presence of RA. Glucose, on the other hand, had minimal effect (**Figure 6A**). Although MLN-DCs had the highest base-line RALDH activity among the three groups tested, neither RA nor glucose had an impact (**Figure 6B**). In contrast, siLP-DCs derived from 2-weeks old neonatal SPF mice which had low basal RALDH activity at steady state, was significantly increased in the presence of glucose alone. While RA itself induced slight increase of this enzyme activity, the highest activity was observed when both RA and glucose was present (**Figure 6C**). Moreover, this RALDH activity promoting effect of glucose was also observed when siLP mixed lymphocytes derived from 8-weeks old adult mice were cultured with glucose for 20 h. Under the culture conditions tested, RA by itself was found to have minimal effect on the already high RALDH activity, whereas the addition of glucose in the media was able to further boost this activity (**Figure 6D**).

Finally, in order to ascertain functional relevance of these findings, we sorted to determine whether supplementation

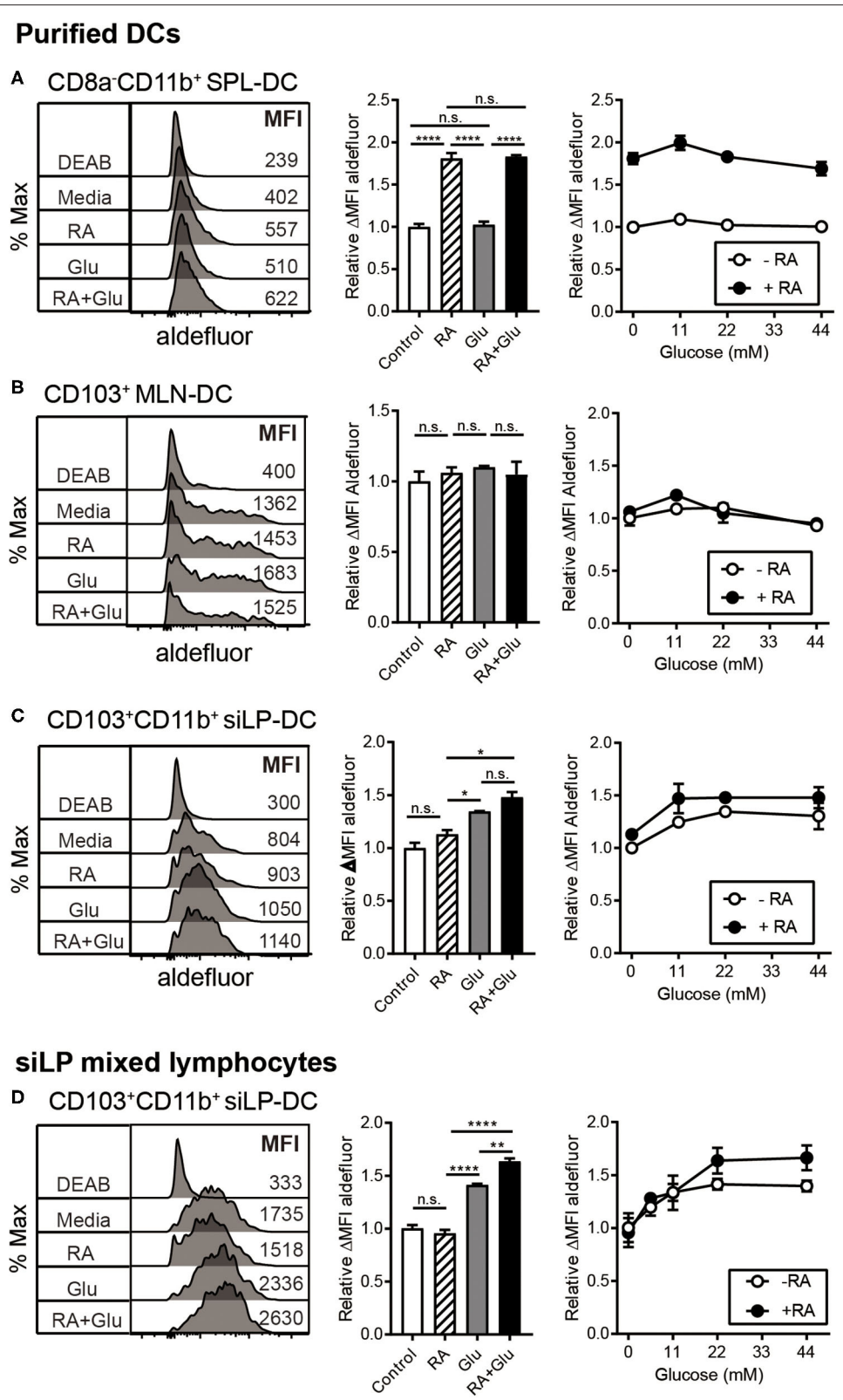


FIGURE 6 | Glucose induces RALDH activity specifically in siLP-DCs. MLN-DCs and Spleen DCs (SPL-DC) from adult SPF mice, or siLP-DCs from 2-weeks old neonatal SPF mice were either purified **(A–C)**, or total single cell suspensions were isolated from adult SPF mice SI **(D)**. Cells were cultured for 20 h in glucose-free

(Continued)

FIGURE 6 | media with or without treatments, following which ALDEFLUOR assay was performed. Fluorescence intensities of aldefluor in $CD8^{-}CD11b^{+}$ SPL-DCs **(A)**, $CD103^{+}$ MLN-DCs **(B)** and $CD103^{+}CD11b^{+}$ siLP-DCs **(C,D)**, were analyzed by flow cytometry. RALDH activity depicted in overlaid histograms and bar graphs are from the cells cultured with glucose (22 mM) or RA (1 nM) or both. The line graphs indicate RALDH activity from the cells cultured with different concentrations of glucose (0, 11, 22, and 44 mM) in the presence or absence of RA (1 nM). Data shown is representative of at least three independent experiments. MEAN ± SEM are indicated. Statistical significance was determined by one-way ANOVA with Turkey's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, n.s., not statistically significant.

of glucose, presumably through the generation of RA, can facilitate the generation of iTreg cells. For this, we performed an *in vitro* iTreg induction assay with magnetically purified $CD11c^{hi}$ DCs (with "high" CD11c expression) that were pre-treated with glucose and incubated with naïve T cells under suboptimal iTreg inducing condition. We observed significant increase in iTreg induction when siLP-DCs were pre-treated with glucose, compared to mock. This effect of glucose pre-treatment was specific for siLP-DCs, and was not observed when MLN-DCs were used in the assay (**Figures 7A,B** and **Figure S4**). These results, in accordance to the results presented in **Figure 6B**, suggested that the siLP DCs are particularly more susceptible to glucose treatment, and thereby, presumably through enhanced RALDH activity, acquire superior iTreg cell induction capacity compared to MLN DCs. It is to be noted however, that while the purified $CD11c^{hi}MHCII^{+}$ DCs used in this assay are present in similar frequencies, there are some site specific differences with regard to the expression of MHCII and CD11c in MLN and siLP. Compared to siLP, MLN has significantly higher proportion of $CD11c^{-}MHCII^{+}$ and $CD11c^{int}MCHII^{-}$ (with "intermediate" CD11c expression) populations (**Figure S4**, left panels). Furthermore, the expression of MHCII in purified MLN DCs is lower than that of siLP DCs (**Figure S4**, right panels). These observations raised the formal possibility that enhanced MHCII expression in siLP, rather than glucose mediated enhanced RALDH activity may be responsible for increased iTreg conversion. However, pre-treatment with RA, either alone or in the presence of glucose, resulted in equally efficient iTreg induction irrespective of the source of the DCs, suggesting that the benefit of glucose pre-treatment is indeed primarily due to enhanced RA production (**Figures 7A,B**). Lastly, when iTreg induction was carried out in vitro in a DC independent manner, supplementation of the media with excess glucose had little effect, thereby further substantiating the role of DC derived RALDH in this process (**Figure S5**).

DISCUSSION

There is accumulating evidence suggesting that vitamin A and its metabolites play a pivotal role in maintaining various biological processes (38, 52, 65–67). Dietary supplementation of RA in the context of cutaneous T cell lymphoma and acute promyelocytic leukemia, have been shown to have beneficial outcome (68–71). Furthermore, many studies highlight anti-inflammatory activities of RA at mucosal sites and tissues, such as intestinal mucosa, airways, lung, central nervous system and skin (72–82). In addition to the effect of RA on cancer and inflammatory diseases, vitamin A or its metabolites play an important role to suppress diet-induced obesity and insulin resistance (83–85). Therefore, a better understanding of the cellular and molecular parameters responsible for vitamin A metabolism is of great biological relevance. In this study by identifying the role of diet, and the importance of glucose consumption for establishing RALDH activity early in life in small intestine DCs, we make substantial contribution to our knowledge related to the role of nutritional components in establishing immunological tolerance at mucosal sites.

By feeding AF diet to mice raised under germ free condition we found that small intestine $CD103^{+}CD11b^{+}$ LP-DCs require a dietary component as an initial trigger for RALDH activity. Since vitamin A in diet is known to be essential to activate RALDH and generate RA in LP-DCs, LP-SCs, MLN-DCs, MLN-SCs, and IECs (40, 43, 44), one explanation of this observation could be the possibility that at steady state, AF mice consume less vitamin A compared to GF mice raised on NCD. However, our further experiments in conjunction with a previous report (50) strongly indicate that the availability of vitamin A and the way it is fed does not account for the low RALDH activity in AF mice. Even supplementing large excess of vitamin A to AFD failed to restore the reduction in RALDH activity in neonatal GF mice weaned on AFD compared to NCD, suggesting that a possible inferior intestinal absorption rate of vitamin A in AF mice is unlikely a cause as well.

In order to define the responsible factor in diet, we employed diet regimes with well-defined compositions. First, by weaning neonate AF mice onto amino acid defined diet in which only the proteins were replaced by defined concentration of amino acids we found that protein macromolecules are dispensable. This was a surprising finding and was not due to any unaccounted protein contamination in the AAD* diet, since the same diet failed to induce small intestinal pTreg cells, a phenomenon known to be dependent on peptide based antigen presentation. Furthermore, subsequent experiments using defined diet regimes eliminated starch and minerals as well. There are distinct differences between the purified protein and starch free diet AAD*_StF and the AFD. First, the diet forms (pellet vs. liquid), second the carbohydrate sources (sucrose vs. glucose) and third the amount of carbohydrate (sucrose 50 vs. glucose 22%). Since the forms of diet as well as the refinement status of NCD can influence experimental outcome (86), we utilized different types of diets (unrefined NCD, purified diets and liquid form of diet) and compared between these groups. In order to address the first issue, we generated a liquid form of diet containing 50% of sucrose (AFD_S500) and compared with the pellet form of AAD*_StF containing around 50% of sucrose. Irrespective of the diet types, both groups showed a similar level of the RALDH activity in LP-DCs and it was

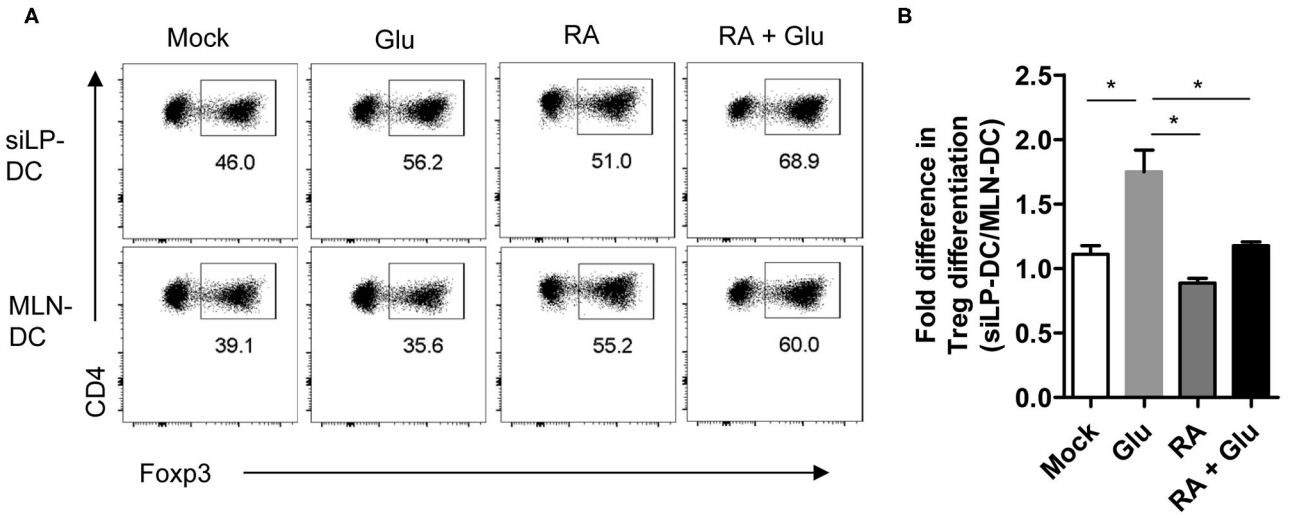


FIGURE 7 | Pretreatment of glucose in siLP-DCs, but not in MLN-DC, significantly induce iTreg cells *in vitro*. siLP and MLN CD11c$^+$ DCs were pre-cultured in glucose-free media or media supplemented with 22 mM glucose (Glu), 1nM RA, or with both glucose and RA. After 14 h the DCs were washed and co-cultured with naïve CD4$^+$ T cells under suboptimal Treg inducing condition. After 72 h Foxp3$^+$ cells was analyzed by FACS. **(A)** Representative FACS plots. **(B)** Bar graphs representing fold differences in Treg induction mediated by siLP-DC vs. MLN-DC. *$P < 0.05$. Data are representative of two independent experiments with similar results.

significantly higher than AFD that contained 22% of glucose. Sucrose is digested to fructose and glucose by the enzyme sucrase that is secreted from the brush border of the small intestine. Furthermore, a recent study reported that at a steady state, most dietary fructose is metabolized and cleared by the small intestine epithelial cells (87). It therefore appeared possible that the metabolites from dietary fructose may have a role in RA metabolism. However, when liquid diet containing 50% sucrose (AFD_S500) vs. 50% glucose (AFD_G500) were compared, we observed comparable RALDH induction; thereby indicating that appropriate concentration of even a monosaccharide unit of dietary glucose is sufficient to initiate RALDH activity in LPDCs. Of note, while in these experiments the relative RALDH activities were calculated on a per cell basis, the variations in total cells obtained from intestinal lamina propria precluded us from reliably calculating the absolute numbers of RALDH expressing intestinal DCs in mice fed with the different dietary regimes.

Although the above experiments were performed primarily on neonatal AF mice in germ free conditions, several lines of evidence strongly suggested that these changes in RALDH activity in response to dietary glucose is also apparent in SPF mice comprised of normal gut microflora. First, while pre-weaned SPF mice, like GF mice, displayed low RALDH activity in siLP-DCs, its activity was increased when they were fed with NCD after weaning. Second, and converse to this observation, when adult SPF mice were fed with AFD (containing 22% glucose) or pre-weaned SPF mice were weaned onto AFD, they displayed significantly lower RALDH activity in siLP-DCs. Third, this reduction in RALDH activity however was not observed when SPF mice were weaned onto AAD, which has increased carbohydrate content compared to AFD. Taken together these results strongly suggest that the glucose dependent changes in RALDH activity in siLP-DCs is still apparent in mice with normal microbiome content and underscores the physiological relevance of this study.

The composition of carbohydrate in NCD is approximately 44∼63% (SPFD and GFD) and 62∼67% in purified diets (AIN-93G and AAD*), which are significantly higher than the carbohydrate content in mother's milk (6.9–7.2% in human and 3% in mouse) (88, 89). On the other hand, AFD contains 22% glucose which is 2.5-fold lower when compared to control diets. The reason behind this is the fact that the glycemic index (GI) of glucose (103 ± 3) is much higher than that of sucrose (65 ± 4) and fructose (15 ± 4) (90). Therefore, the percentage composition of glucose constituting AFD was calculated by factoring in its GI as a parameter in order to maintain mice healthy and capable of breeding. Indeed, from our previous report and earlier studies (91–93), mice raised in AFD were healthy and normal in size and weight, and displayed no sign of nutritional deficiency. Despite these apparent normalcy however, in this study we found that additional dietary source of carbohydrate is required to trigger LP-DC RALDH activity in AFD fed mice.

As a functional consequence we found that pre-treatment with glucose results in enhanced iTreg inducing property specifically in siLP-DCs in an *in vitro* assay system. This finding is in concert with our previous finding where, in a OTII CD4$^+$ T cell transfer based *in vivo* assay we observed significantly reduced pTreg generation in AF, compared to SPF and GF mice (50). We propose that the primary cause of such reduced pTreg conversion if AF mice despite being fed comparable amount of OVA to that in SPF and GF mice, is the lower amount of dietary

carbohydrate content in AFD. Taken together our study uncovers the functional significance of minute refinements in dietary compositions and underscores the fact that subtle differences in diet may have significant influence on immune composition and overall organismal physiology.

The role of glycolysis on DC physiology is well-appreciated (94, 95). A recent study further suggests that the level of glucose consumed is capable of reprograming glycolytic metabolism and mitochondrial oxidative phosphorylation in lipopolysaccharide (LPS)-stimulated DCs and repress pro-inflammatory responses (96). The authors propose that the extent of glucose consumption can control the expression of glucose transporters and glycolytic enzymes by mammalian target of rapamycin complex 1 (mTORC1) / hypoxia-inducible factor 1a (HIF1a) / inducible nitric oxide synthase (iNOS) signaling circuit (97–99). There are several studies dating as far back to the late 1950's that point to a possible connection between glucose consumption and vitamin A metabolism (100). In light of these previous reports, in this study we have successfully established an unequivocal role of dietary glucose consumption and RALDH activity in LP-DCs and therefore intestinal tolerance. Precise molecular nature of this connection, and how it affects specifically the LP-DCs, remain less understood, and will be an active area of our investigation in recent future. In sum, these findings significantly enhance our knowledge on the crosstalk between functional relationship between immune homeostasis and dietary intake and expand our understanding of therapeutic strategies for clinical applications.

MATERIALS AND METHODS

Mice

Specific pathogen-free (SPF) C57BL/6 (B6) mice were purchased from The Jackson Laboratory and maintained in the animal facility of POSTECH Biotech Center. A colony of Germ-free (GF) B6 mice was established at POSTECH from breeders obtained from Dr. Andrew Macpherson. GF B6 mice were maintained in sterile flexible film isolators (Class Biological Clean Ltd., USA) and GF status was regularly monitored by culturing feces of GF mice. AF mice were generated and maintained in sterile flexible film isolator as previously described (50). All animal studies were performed in accordance with the guidelines of Institutional Animal Care and Use Committee of POSTECH.

Mouse Diets

SPF diet (38057, Purina Lab) and GF diet (2018S, Envigo) were used as normal chow diet (NCD) for the maintenance of SPF and GF mice at the animal facility of POSTECH Biotech Center. AF diet (AFD) was self-generated as previously described (50). The custom diets were generated and supplied from Envigo: amino acid-defined diet (AAD, TD.01084), AAD with 3× vitamin A (AAD*, TD.160107), starch deficient-AAD* (AAD*_StF, TD.160108), and mineral mix powder (TD.94049). AFD-based diets were self-generated as follows: 22% glucose in AFD (AFD_G220, same as general AFD), 50% glucose in AFD (AFD_G500) and 50% sucrose in AFD (AFD_S500).

Preparation and Processing of Single Cell Suspensions From Small Intestine, MLN, and Spleen

Single cell suspension from SI was prepared and processed as previously described (50). In brief, SIs were dissected and opened longitudinally after removal of Peyer's patches. Tissues were cut into pieces and incubated in PBS buffer containing 3% fetal bovine serum (FBS), 10mM EDTA, 20 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1mM sodium pyruvate with gentle stirring at 37°C for 20 min to strip intestinal epithelial cells (IECs). SI segments were collected by strainer for isolating lamina propria lymphocytes and IECs in the media were enriched by 25–40 % Percoll density gradient centrifugation. Collected SI segments were digested with 400 Mandl units/ml collagenase D (Roche, Cat no. 11088882001) and 10 μg/ml DNase I (Sigma, 04536282001) in RPMI 1640 containing 3% fetal bovine serum (FBS), 20 mM EDTA, 20 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate and 1mM Non-essential amino acids at 37°C for 45 min with continuous stirring. To stop the enzyme digestion, EDTA was added (10 mM final concentration) and cells were further incubated for 5 min at 37°C. Cell suspensions were enriched by 40–75% Percoll density gradient centrifugation. MLNs and spleens were harvested and minced with razor blade, followed by digesting at 37°C for 20–30 min with enzyme digestion buffer that were used for SI cell preparation. For analysis of stroma cells from SI, the enzyme digestions were repeated two more times with fresh enzymes for 20 min. The remaining procedures were same as SI cell preparation.

ALDEFLUOR Assay and Flow Cytometry

Aldehyde dehydrogenate (ALDH) activity in individual cells was measured using ALDEFLUOR kits (StemCell Technologies), according to the manufacturer's protocol with modifications. Subsequently, aldefluor-reacted cells were stained with antibodies and analyzed by flow cytometry. CD16/32 Fc Blocker (93, Cat. No. 11302), CD3e-PerCp-Cy5.5 (145-2C11, Cat. No. 100328), Thy1.2-PerCp-Cy5.5 (53-2.1, Cat. No. 140322), B220-PerCp-Cy5.5 (RA3-6B2, Cat. No. 103236), NK1.1-PerCp-Cy5.5 (PK136, Cat. No. 108728), MHC class II-eFluor 450 (M5/114.15.2, Cat. No. 48-5321-82), CD11c-PE-Cy7 (N418, Cat. No. 25-0114-82), CD103-PE (2E7, Cat. No. 12-1031-82), CD11b-APC (M1/70, Cat. No. 17-0112-82), and CD8-BV650 (53-6.7, Cat. No. 100742).

Fluorochrome-conjugated antibodies for single-cell staining were purchased from BD Biosceineces, BioLegend, Thermo Fisher Scientific and Tonbo Biosciences. For LP-DC phenotypic analysis, cells were stained with antibodies and analyzed by flow cytometry: CD16/32 Fc Blocker, CD3e-PerCp-Cy5.5, Thy1.2-PerCp-Cy5.5, B220-PerCp-Cy5.5, NK1.1-PerCp-Cy5.5, CD11c-PE-Cy7, MHC class II-APC-eFluor 780 (M5/114.15.2, Cat. No. 47-5321-82), CD103-BV510 (2E7, Cat. No. 121423) and CD11b-FITC (M1/70, Cat. No.35-0112), Siglec-F-Alex Fluor 647 (E50-2440, Cat. No. 562680), Ly6C-PB (HK1.4, Cat. No. 128014), and CX3CR1-PE (SA011F11, Cat. No. 149006). For IECs and LP-SCs analysis, cells were stained with CD45-PerCp-Cy5.5 (30-F11, Cat.

No. 103132), EpCAM1-eFluor 450 (G8.8, Cat. No. 48-5791-82), Pdpn-PE (8.1.1, Cat. No. 127408), and CD31-APC (390, Cat. No. 102410). For intracellular cytokine staining, cells were stimulated with eBioscience cell stimulation cocktail plus protein transport inhibitors (Thermo Fisher Scientific) for 4 h. Stimulated cells were stained with antibodies for surface markers followed by fixation and permeabilization with manufacturer's instruction (BD Biosciences). Intracellular staining with Foxp3, Tbet, and GATA3 were performed by eBioscience Foxp3/Transcription factor staining buffer set (Thermo Fisher Scientific): CD4-APC-Cy7 (RM4-5, Cat. No. 25-0042), TCR-β-PE-Cy7 (H57-597, Cat. No. 25-5961-82), CD44-eFluor 450 (IM7, Cat. No. 48-0441-82), CD62L-FITC (MEL-14, Cat. No. 104406), Nrp-1-PE (3E12, Cat. No. 145204), Foxp3-PE-Cy5.5 (FJK-16s, Cat. No. 35-5773-82), GATA3-PE (TWAJ, Cat. No. 12-9966-42), Tbet-APC (4B10, Cat. No. 644814). Dead cells were excluded by Ghost Dye (Tonbo Biosciences) or Propidium iodide (PI, Sigma) staining. Stained cells were analyzed by LSRFortessa or FACSCantoII (BD Biosciences) and data were analyzed by FlowJo software (Tree Star).

RNA Isolatio n and Real-Time PCR

$CD11c^+MHC\text{-}II^+CD103^+CD11b^+$ siLP-DCs were sorted with a Moflo XDP (Beckman Coulter) and RNA was extracted using TriZol (Invitrogen). IECs were obtained after stripping SI by EDTA and RNA was extracted using RNeasy Mini kit (Qiagen) or Hybrid-R^{TM} (GeneAll). Complementary DNA was generated using QuantiTect Reverse Transcription Kit (Qiagen) or Improm II Reverse Transcriptase (Promega). Target messenger RNA was quantified using SYBR green master mix (Applied Biosystems or Takara) and gene specific primers in a duplex reaction with Hprt (TaqMan gene expression assays, Thermo Fisher Scientific): Hprt (Mm01545399_m1), (Aldh1a1 Mm00657317_m1), Aldh1a2 (Mm00501306_m1), Ido1 (Mm00492586_m1), and Tgfb1 (Mm01178820_m1). Data were analyzed on Applied Biosystems ViiA^{TM} 7 Real-Time PCR system (Applied Biosystems) or Rotor-Gene Q (Qiagen). Changes in gene expression were calculated by the comparative C_T method and fold changes were calculated using $2^{-\Delta\Delta Ct}$ method.

In vitro Experiments

Common RPMI 1640 Medium (Gibco, 11875119) contains 11mM glucose, but RPMI 1640 Medium, no glucose (Gibco, 11879020) does not contain glucose. Retinoic acid (RA, Sigma) and Glucose (Sigma) were treated to culture cells with a various concentration. SiLP-DCs, MLN-DCs and SPL-DCs were purified using anti-CD11c magnetic beads and MACS columns (Milteny Biotec). Purified DCs and SI cell suspensions (2×10^5 per well in a 96-well plate) were cultured in glucose-free media with different concentration of glucose (0, 11, 22, and 44 mM) in the presence or absence of RA (1 nM). After overnight culture (20 h), cells were analyzed for RALDH activity using ALDEFLUOR assay kit. For *in vitro* Treg differentiation assay, DCs from siLP or MLN were purified in the same way as above. Purified DCs (2×10^4 per well in a 96-well plate) were cultured in glucose-free media with glucose (22 mM) in the presence or absence of RA (1 nM) for 14 h. Then, they were co-cultured with 2×10^5 naïve $CD4^+$ T cells in the presence of 0.1 μg/ml anti-CD3, 0.1 ng/ml TGFβ-1 and 100 U IL-2. Cultures were incubated for a total of 3 days followed by FACS analysis.

Statistical Analyses

Mean and SEM values were calculated. Two-tailed unpaired *t*-tests and one-way or two-way ANOVA with Turkey's multiple comparison test were performed, with GraphPad Prism Software.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of POSTECH.

AUTHOR CONTRIBUTIONS

H-JK and S-WH designed, performed experiments, and analyzed data. RV performed *in vitro* iTreg differentiation experiments and analyzed data. JJ, ML, NK, and DK performed isolation of intestinal epithelial and lamina propria cells. KK performed gene expression experiments on intestinal DCs and analyzed data. CS along with H-JK initiated the study and supervised it in the initial days. DR and S-HI supervised and finalized the study to its completion. H-JK, S-HI, and DR wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Institute for Basic Science [Project Code: IBS-R005-D1], POSCO GREEN SCIENCE Research Fund [Project no. 2019Y073], and ImmunoBiome Inc [Project no. IMB-2020-OR101].

ACKNOWLEDGMENTS

We thank Tae-Kyu Kim, Jung-Wook Seo, Sung-Hwan Na, Hee-Jung Woo, and Hae-Jin Jung for technical support; Jonathan Sprent, You Jeong Lee, and Jae-Ho Cho for discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01897/full#supplementary-material

REFERENCES

1. Bekiaris V, Persson EK, Agace WW. Intestinal dendritic cells in the regulation of mucosal immunity. *Immunol Rev.* (2014) 260:86–101. doi: 10.1111/imr.12194
2. Joeris T, Muller-Luda K, Agace WW, Mowat AM. Diversity and functions of intestinal mononuclear phagocytes. *Mucosal Immunol.* (2017) 10:845–64. doi: 10.1038/mi.2017.22
3. Stagg AJ. Intestinal dendritic cells in health and gut inflammation. *Front Immunol.* (2018) 9:2883. doi: 10.3389/fimmu.2018.02883
4. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. *Nat Immunol.* (2011) 12:5–9. doi: 10.1038/ni0111-5
5. Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. *Nature.* (2016) 535:65–74. doi: 10.1038/nature18847
6. Veldhoen M, Brucklacher-Waldert V. Dietary influences on intestinal immunity. *Nat Rev Immunol.* (2012) 12:696–708. doi: 10.1038/nri3299
7. Ma N, Guo P, Zhang J, He T, Kim SW, Zhang G, et al. Nutrients mediate intestinal bacteria-mucosal immune crosstalk. *Front Immunol.* (2018) 9:5. doi: 10.3389/fimmu.2018.00005
8. Schlitzer A, Mcgovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity.* (2013) 38:970–83. doi: 10.1016/j.immuni.2013.04.011
9. Esterhazy D, Loschko J, London M, Jove V, Oliveira TY, Mucida D. Classical dendritic cells are required for dietary antigen-mediated induction of peripheral T(reg) cells and tolerance. *Nat Immunol.* (2016) 17:545–55. doi: 10.1038/ni.3408
10. Nutsch K, Chai JN, Ai TL, Russler-Germain E, Feehley T, Nagler CR, et al. Rapid and efficient generation of regulatory T cells to commensal antigens in the periphery. *Cell Rep.* (2016) 17:206–20. doi: 10.1016/j.celrep.2016.08.092
11. Aychek T, Mildner A, Yona S, Kim KW, Lampl N, Reich-Zeliger S, et al. IL-23-mediated mononuclear phagocyte crosstalk protects mice from Citrobacter rodentium-induced colon immunopathology. *Nat Commun.* (2015) 6:6525. doi: 10.1038/ncomms7525
12. Mayer JU, Demiri M, Agace WW, Macdonald AS, Svensson-Frej M, Milling SW. Different populations of CD11b(+) dendritic cells drive Th2 responses in the small intestine and colon. *Nat Commun.* (2017) 8:15820. doi: 10.1038/ncomms15820
13. Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol.* (2009) 124:3–20; quiz 21–22. doi: 10.1016/j.jaci.2009.05.038
14. Macia L, Thorburn AN, Binge LC, Marino E, Rogers KE, Maslowski KM, et al. Microbial influences on epithelial integrity and immune function as a basis for inflammatory diseases. *Immunol Rev.* (2012) 245:164–76. doi: 10.1111/j.1600-065X.2011.01080.x
15. Nishida A, Inoue R, Inatomi O, Bamba S, Naito Y, Andoh A. Gut microbiota in the pathogenesis of inflammatory bowel disease. *Clin J Gastroenterol.* (2018) 11:1–10. doi: 10.1007/s12328-017-0813-5
16. Sugihara K, Morhardt TL, Kamada N. The role of dietary nutrients in inflammatory bowel disease. *Front Immunol.* (2018) 9:3183. doi: 10.3389/fimmu.2018.03183
17. Sanz Y, De Pama G, Laparra M. Unraveling the ties between celiac disease and intestinal microbiota. *Int Rev Immunol.* (2011) 30:207–18. doi: 10.3109/08830185.2011.599084
18. Berin MC, Sampson HA. Mucosal immunology of food allergy. *Curr Biol.* (2013) 23:R389–400. doi: 10.1016/j.cub.2013.02.043
19. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol.* (2008) 8:435–46. doi: 10.1038/nri2335
20. Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, et al. Origin of the lamina propria dendritic cell network. *Immunity.* (2009) 31:513–25. doi: 10.1016/j.immuni.2009.08.010
21. Rescigno M, Di Sabatino A. Dendritic cells in intestinal homeostasis and disease. *J Clin Invest.* (2009) 119:2441–50. doi: 10.1172/JCI39134
22. Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity.* (2009) 31:502–12. doi: 10.1016/j.immuni.2009.06.025
23. Rescigno M. Intestinal dendritic cells. *Adv Immunol.* (2010) 107:109–38. doi: 10.1016/B978-0-12-381300-8.00004-6
24. Yokota A, Takeuchi H, Maeda N, Ohoka Y, Kato C, Song SY, et al. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int Immunol.* (2009) 21:361–77. doi: 10.1093/intimm/dxp003
25. Villablanca EJ, Wang S, De Calisto J, Gomes DC, Kane MA, Napoli JL, et al. MyD88 and retinoic acid signaling pathways interact to modulate gastrointestinal activities of dendritic cells. *Gastroenterology.* (2011) 141:176–85. doi: 10.1053/j.gastro.2011.04.010
26. Iwata M, Eshima Y, Kagechika H. Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors. *Int Immunol.* (2003) 15:1017–25. doi: 10.1093/intimm/dxg101
27. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity.* (2004) 21:527–38. doi: 10.1016/j.immuni.2004.08.011
28. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science.* (2006) 314:1157–60. doi: 10.1126/science.1132742
29. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science.* (2007) 317:256–60. doi: 10.1126/science.1145697
30. Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood.* (2008) 111:1013–20. doi: 10.1182/blood-2007-06-096438
31. Cha HR, Chang SY, Chang JH, Kim JO, Yang JY, Kim CH, et al. Downregulation of Th17 cells in the small intestine by disruption of gut flora in the absence of retinoic acid. *J Immunol.* (2010) 184:6799–806. doi: 10.4049/jimmunol.0902944
32. Depaolo RW, Abadie V, Tang F, Fehlner-Peach H, Hall JA, Wang W, et al. Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature.* (2011) 471:220–4. doi: 10.1038/nature09849
33. Mielke LA, Jones SA, Raverdeau M, Higgs R, Stefanska A, Groom JR, et al. Retinoic acid expression associates with enhanced IL-22 production by gammadelta T cells and innate lymphoid cells and attenuation of intestinal inflammation. *J Exp Med.* (2013) 210:1117–24. doi: 10.1084/jem.20121588
34. Takeuchi H, Yokota-Nakatsuma A, Ohoka Y, Kagechika H, Kato C, Song SY, et al. Retinoid X receptor agonists modulate Foxp3(+) regulatory T cell and Th17 cell differentiation with differential dependence on retinoic acid receptor activation. *J Immunol.* (2013) 191:3725–33. doi: 10.4049/jimmunol.1300032
35. Spencer SP, Wilhelm C, Yang Q, Hall JA, Bouladoux N, Boyd A, et al. Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science.* (2014) 343:432–7. doi: 10.1126/science.1247606
36. Brown CC, Esterhazy D, Sarde A, London M, Pullabhatla V, Osma-Garcia I, et al. Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program. *Immunity.* (2015) 42:499–511. doi: 10.1016/j.immuni.2015.02.003
37. Kim MH, Taparowsky EJ, Kim CH. Retinoic acid differentially regulates the migration of innate lymphoid cell subsets to the gut. *Immunity.* (2015) 43:107–19. doi: 10.1016/j.immuni.2015.06.009
38. Erkelens MN, Mebius RE. Retinoic acid and immune homeostasis: a balancing act. *Trends Immunol.* (2017) 38:168–80. doi: 10.1016/j.it.2016.12.006
39. Zaman TS, Arimochi H, Maruyama S, Ishifune C, Tsukumo SI, Kitamura A, et al. Notch balances Th17 and induced regulatory T cell functions in dendritic cells by regulating Aldh1a2 expression. *J Immunol.* (2017) 199:1989–97. doi: 10.4049/jimmunol.1700645
40. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, De Vos AF, O'toole T, et al. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells gut-draining lymph node stromal cells is controlled by dietary vitamin A. *J Immunol.* (2011) 186:1934–42. doi: 10.4049/jimmunol.1001672
41. Mcdonald KG, Leach MR, Brooke KW, Wang C, Wheeler LW, Hanly EK, et al. Epithelial expression of the cytosolic retinoid chaperone cellular retinol binding protein II is essential for in vivo imprinting of local gut dendritic cells by lumenal retinoids. *Am J Pathol.* (2012) 180:984–97. doi: 10.1016/j.ajpath.2011.11.009

42. Bimczok D, Kao JY, Zhang M, Cochrun S, Mannon P, Peter S, et al. Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells. *Mucosal Immunol.* (2015) 8:533–44. doi: 10.1038/mi.2014.86
43. Vicente-Suarez I, Larange A, Reardon C, Matho M, Feau S, Chodaczek G, et al. Unique lamina propria stromal cells imprint the functional phenotype of mucosal dendritic cells. *Mucosal Immunol.* (2015) 8:141–51. doi: 10.1038/mi.2014.51
44. Goverse G, Molenaar R, Macia L, Tan J, Erkelens MN, Konijn T, et al. Diet-derived short chain fatty acids stimulate intestinal epithelial cells to induce mucosal tolerogenic dendritic cells. *J Immunol.* (2017) 198:2172–81. doi: 10.4049/jimmunol.1600165
45. Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, et al. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. *Mucosal Immunol.* (2013) 6:847–56. doi: 10.1038/mi.2012.123
46. Zeng R, Bscheider M, Lahl K, Lee M, Butcher EC. Generation and transcriptional programming of intestinal dendritic cells: essential role of retinoic acid. *Mucosal Immunol.* (2016) 9:183–93. doi: 10.1038/mi.2015.50
47. Stock A, Booth S, Cerundolo V. Prostaglandin E2 suppresses the differentiation of retinoic acid-producing dendritic cells in mice and humans. *J Exp Med.* (2011) 208:761–73. doi: 10.1084/jem.20101967
48. Zhu B, Buttrick T, Bassil R, Zhu C, Olah M, Wu C, et al. IL-4 and retinoic acid synergistically induce regulatory dendritic cells expressing Aldh1a2. *J Immunol.* (2013) 191:3139–51. doi: 10.4049/jimmunol.1300329
49. Ohoka Y, Yokota-Nakatsuma A, Maeda N, Takeuchi H, Iwata M. Retinoic acid and GM-CSF coordinately induce retinal dehydrogenase 2 (RALDH2) expression through cooperation between the RAR/RXR complex and Sp1 in dendritic cells. *PLoS ONE.* (2014) 9:e96512. doi: 10.1371/journal.pone.0096512
50. Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, et al. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science.* (2016) 351:858–63. doi: 10.1126/science.aac5560
51. Agace WW, Persson EK. How vitamin A metabolizing dendritic cells are generated in the gut mucosa. *Trends Immunol.* (2012) 33:42–8. doi: 10.1016/j.it.2011.10.001
52. Oliveira LM, Teixeira FME, Sato MN. Impact of retinoic acid on immune cells and inflammatory diseases. *Mediators Inflamm.* (2018) 2018:3067126. doi: 10.1155/2018/3067126
53. Pezoldt J, Pasztoi M, Zou M, Wiechers C, Beckstette M, Thierry GR, et al. Neonatally imprinted stromal cell subsets induce tolerogenic dendritic cells in mesenteric lymph nodes. *Nat Commun.* (2018) 9:3903. doi: 10.1038/s41467-018-06423-7
54. Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, et al. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci USA.* (1999) 96:9118–23. doi: 10.1073/pnas.96.16.9118
55. Moreb JS, Ucar D, Han S, Amory JK, Goldstein AS, Ostmark B, et al. The enzymatic activity of human aldehyde dehydrogenases 1A2 and 2 (ALDH1A2 and ALDH2) is detected by Aldefluor, inhibited by diethylaminobenzaldehyde and has significant effects on cell proliferation and drug resistance. *Chem Biol Interact.* (2012) 195:52–60. doi: 10.1016/j.cbi.2011.10.007
56. Mueller SN, Germain RN. Stromal cell contributions to the homeostasis and functionality of the immune system. *Nat Rev Immunol.* (2009) 9:618–29. doi: 10.1038/nri2588
57. Roozendaal R, Mebius RE. Stromal cell-immune cell interactions. *Annu Rev Immunol.* (2011) 29:23–43. doi: 10.1146/annurev-immunol-031210-101357
58. Frota-Ruchon A, Marcinkiewicz M, Bhat PV. Localization of retinal dehydrogenase type 1 in the stomach and intestine. *Cell Tissue Res.* (2000) 302:397–400. doi: 10.1007/s004410000281
59. Lampen A, Meyer S, Arnhold T, Nau H. Metabolism of vitamin A and its active metabolite all-trans-retinoic acid in small intestinal enterocytes. *J Pharmacol Exp Ther.* (2000) 295:979–85. Available online at: http://jpet.aspetjournals.org/content/295/3/979
60. Thomas S, Prabhu R, Balasubramanian KA. Retinoid metabolism in the rat small intestine. *Br J Nutr.* (2005) 93:59–63. doi: 10.1079/BJN20041306
61. Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol.* (2009) 2:340–50. doi: 10.1038/mi.2009.13
62. Haselbeck RJ, Hoffmann I, Duester G. Distinct functions for Aldh1 and Raldh2 in the control of ligand production for embryonic retinoid signaling pathways. *Dev Genet.* (1999) 25:353–64. doi: 10.1002/(SICI)1520-6408(1999)25:4<353::AID-DVG9>3.0.CO;2-G
63. Grun F, Hirose Y, Kawauchi S, Ogura T, Umesono K. Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J Biol Chem.* (2000) 275:41210–8. doi: 10.1074/jbc.M007376200
64. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto De Lafaille MA, Parkhurst CN, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med.* (2012) 209:1723–42, S1721. doi: 10.1084/jem.20120914
65. Zhang Z, Li J, Zheng W, Zhao G, Zhang H, Wang X, et al. Peripheral lymphoid volume expansion and maintenance are controlled by gut microbiota via RALDH+ dendritic cells. *Immunity.* (2016) 44:330–42. doi: 10.1016/j.immuni.2016.01.004
66. Widjaja-Adhi MAK, Palczewski G, Dale K, Knauss EA, Kelly ME, Golczak M, et al. Transcription factor ISX mediates the cross talk between diet and immunity. *Proc Natl Acad Sci USA.* (2017) 114:11530–5. doi: 10.1073/pnas.1714963114
67. Kim CH. Control of innate and adaptive lymphocytes by the RAR-retinoic acid axis. *Immune Netw.* (2018) 18:e1. doi: 10.4110/in.2018.18.e1
68. Tallman MS, Andersen JW, Schiffer CA, Appelbaum FR, Feusner JH, Woods WG, et al. All-trans retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. *Blood.* (2002) 100:4298–302. doi: 10.1182/blood-2002-02-0632
69. Paydas S, Yavuz S, Disel U, Sahin B, Canbolat T, Tuncer I. Vasculitis associated with all trans retinoic acid (ATRA) in a case with acute promyelocytic leukemia. *Leuk Lymphoma.* (2003) 44:547–8. doi: 10.1080/1042819021000055101
70. Hamada T, Sugaya M, Tokura Y, Ohtsuka M, Tsuboi R, Nagatani T, et al. Phase I/II study of the oral retinoid X receptor agonist bexarotene in Japanese patients with cutaneous T-cell lymphomas. *J Dermatol.* (2017) 44:135–42. doi: 10.1111/1346-8138.13542
71. Sato Y, Fujimura T, Kambayashi Y, Hashimoto A, Aiba S. Successful treatment of advanced primary cutaneous peripheral T-cell lymphoma with oral bexarotene monotherapy. *Case Rep Oncol.* (2018) 11:212–5. doi: 10.1159/000488236
72. Rigopoulos D, Ioannides D, Kalogeromitros D, Katsambas AD. Comparison of topical retinoids in the treatment of acne. *Clin Dermatol.* (2004) 22:408–11. doi: 10.1016/j.clindermatol.2004.03.013
73. Goswami S, Angkasekwinai P, Shan M, Greenlee KJ, Barranco WT, Polikepahad S, et al. Divergent functions for airway epithelial matrix metalloproteinase 7 and retinoic acid in experimental asthma. *Nat Immunol.* (2009) 10:496–503. doi: 10.1038/ni.1719
74. Soroosh P, Doherty TA, Duan W, Mehta AK, Choi H, Adams YF, et al. Lung-resident tissue macrophages generate Foxp3+ regulatory T cells and promote airway tolerance. *J Exp Med.* (2013) 210:775–88. doi: 10.1084/jem.20121849
75. Sakamoto H, Koya T, Tsukioka K, Shima K, Watanabe S, Kagamu H, et al. The effects of all-trans retinoic acid on the induction of oral tolerance in a murine model of bronchial asthma. *Int Arch Allergy Immunol.* (2015) 167:167–76. doi: 10.1159/000437326
76. Bhattacharya N, Yuan R, Prestwood TR, Penny HL, Dimaio MA, Reticker-Flynn NE, et al. Normalizing microbiota-induced retinoic acid deficiency stimulates protective CD8(+) T cell-mediated *immunity* in colorectal cancer. *Immunity.* (2016) 45:641–55. doi: 10.1016/j.immuni.2016.08.008
77. Penny HL, Prestwood TR, Bhattacharya N, Sun F, Kenkel JA, Davidson MG, et al. Restoring retinoic acid attenuates intestinal inflammation and tumorigenesis in APCMin/+ mice. *Cancer Immunol Res.* (2016) 4:917–26. doi: 10.1158/2326-6066.CIR-15-0038
78. Riahi RR, Bush AE, Cohen PR. Topical retinoids: therapeutic mechanisms in the treatment of photodamaged skin. *Am J Clin Dermatol.* (2016) 17:265–76. doi: 10.1007/s40257-016-0185-5

79. Khalil S, Bardawil T, Stephan C, Darwiche N, Abbas O, Kibbi AG, et al. Retinoids: a journey from the molecular structures and mechanisms of action to clinical uses in dermatology and adverse effects. *J Dermatolog Treat.* (2017) 28:684–96. doi: 10.1080/09546634.2017.1309349
80. Takamura R, Watamura N, Nikkuni M, Ohshima T. All-trans retinoic acid improved impaired proliferation of neural stem cells and suppressed microglial activation in the hippocampus in an Alzheimer's mouse model. *J Neurosci Res.* (2017) 95:897–906. doi: 10.1002/jnr.23843
81. Parastouei K, Mirshafiey A, Eshraghian MR, Shiri-Shahsavar MR, Solaymani-Mohammadi F, Chahardoli R, et al. The effect of 1, 25(OH)2 D3 (calcitriol) alone and in combination with all-trans retinoic acid on ROR-gammat, IL-17, TGF-beta, and FOXP3 gene expression in experimental autoimmune encephalomyelitis. *Nutr Neurosci.* (2018) 21:210–8. doi: 10.1080/1028415X.2016.1263039
82. Stebbins MJ, Lippmann ES, Faubion MG, Daneman R, Palecek SP, Shusta EV. Activation of RARalpha, RARgamma, or RXRalpha increases barrier tightness in human induced pluripotent stem cell-derived brain endothelial cells. *Biotechnol J.* (2018) 13:1700093. doi: 10.1002/biot.201700093
83. Ziouzenkova O, Orasanu G, Sharlach M, Akiyama TE, Berger JP, Viereck J, et al. Retinaldehyde represses adipogenesis and diet-induced obesity. *Nat Med.* (2007) 13:695–702. doi: 10.1038/nm1587
84. Berry DC, Noy N. All-trans-retinoic acid represses obesity and insulin resistance by activating both peroxisome proliferation-activated receptor beta/delta and retinoic acid receptor. *Mol Cell Biol.* (2009) 29:3286–96. doi: 10.1128/MCB.01742-08
85. Dalmas E, Lehmann FM, Dror E, Wueest S, Thienel C, Borsigova M, et al. Interleukin-33-Activated Islet-Resident Innate Lymphoid Cells Promote Insulin Secretion through Myeloid Cell Retinoic Acid Production. *Immunity.* (2017) 47:928–42 e927. doi: 10.1016/j.immuni.2017.10.015
86. Pellizzon MA, Ricci MR. The common use of improper control diets in diet-induced metabolic disease research confounds data interpretation: the fiber factor. *Nutr Metab (Lond).* (2018) 15:3. doi: 10.1186/s12986-018-0256-0
87. Jang C, Hui S, Lu W, Cowan AJ, Morscher RJ, Lee G, et al. The small intestine converts dietary fructose into glucose and organic acids. *Cell Metab.* (2018) 27:351–61 e353. doi: 10.1016/j.cmet.2017.12.016
88. Webb B, Johnson A, Alford J. *Fundamentals of Diary Chemistry*. Westport, CT.: AVI Publishing (1974).
89. Ramani S, Stewart CJ, Laucirica DR, Ajami NJ, Robertson B, Autran CA, et al. Human milk oligosaccharides, milk microbiome and infant gut microbiome modulate neonatal rotavirus infection. *Nat Commun.* (2018) 9:5010. doi: 10.1038/s41467-018-07476-4
90. Atkinson FS, Foster-Powell K, Brand-Miller JC. International tables of glycemic index and glycemic load values: 2008. *Diabetes Care.* (2008) 31:2281–3. doi: 10.2337/dc08-1239
91. Pereira P, Forni L, Larsson EL, Cooper M, Heusser C, Coutinho A. Autonomous activation of B and T cells in antigen-free mice. *Eur J Immunol.* (1986) 16:685–8. doi: 10.1002/eji.1830160616
92. Pleasants JR, Johnson MH, Wostmann BS. Adequacy of chemically defined, water-soluble diet for germfree BALB/c mice through successive generations and litters. *J Nutr.* (1986) 116:1949–64. doi: 10.1093/jn/116.10.1949
93. Wostmann BS, Pleasants JR. The germ-free animal fed chemically defined diet: a unique tool. *Proc Soc Exp Biol Med.* (1991) 198:539–46. doi: 10.3181/00379727-198-43286D
94. Pearce EJ, Everts B. Dendritic cell metabolism. *Nat Rev Immunol.* (2015) 15:18–29. doi: 10.1038/nri3771
95. O'neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med.* (2016) 213:15–23. doi: 10.1084/jem.20151570
96. Lawless SJ, Kedia-Mehta N, Walls JF, Mcgarrigle R, Convery O, Sinclair LV, et al. Glucose represses dendritic cell-induced T cell responses. *Nat Commun.* (2017) 8:15620. doi: 10.1038/ncomms15620
97. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med.* (2011) 208:1367–76. doi: 10.1084/jem.20110278
98. Everts B, Amiel E, Van Der Windt GJ, Freitas TC, Chott R, Yarasheski KE, et al. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood.* (2012) 120:1422–31. doi: 10.1182/blood-2012-03-419747
99. Katholnig K, Linke M, Pham H, Hengstschlager M, Weichhart T. Immune responses of macrophages and dendritic cells regulated by mTOR signalling. *Biochem Soc Trans.* (2013) 41:927–33. doi: 10.1042/BST20130032
100. Ray A, Sadhu DP. Carbohydrate metabolism in hypervitaminosis A. *Nature.* (1959) 184 (Suppl. 17):1323. doi: 10.1038/1841323a0

Conflict of Interest: S-HI is the CEO of the ImmunoBiome, but declares no conflicts of interest for this paper.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 23 September 2020
doi: 10.3389/fimmu.2020.02150

Low-Dose LPS Induces Tolerogenic Treg Skewing in Asthma

*Fengxia Ding[1,2,3,4], Bo Liu[2,5], Chao Niu[1,2,3,4], Ting Wang[1,2,3,4], Yaping Wang[1,2,3,4], Gang Geng[1,2,3,4], Daiyin Tian[1,2,3,4], Jihong Dai[1,2,3,4] and Zhou Fu[1,2,3,4]**

[1] Department of Pediatric Respiratory Medicine, Children's Hospital of Chongqing Medical University, Chongqing, China, [2] Ministry of Education Key Laboratory of Child Development and Disorders, Chongqing, China, [3] China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Chongqing, China, [4] Chongqing Key Laboratory of Child Infection and Immunity, Chongqing, China, [5] Department of Cardiothoracic Surgery, Children's Hospital of Chongqing Medical University, Chongqing, China

The mechanism(s) underlying endotoxin tolerance in asthma remain elusive. As the endotoxin lipopolysaccharide (LPS) affects the expression of the regulatory T-cell (Treg)-suppressive glucocorticoid-induced tumor necrosis factor receptor ligand (GITRL) on antigen-presenting dendritic cells (DCs), we hypothesized that LPS-induced changes in DC GITRL expression may impact Treg-mediated T-helper (Th) cell suppression and the induction of endotoxin tolerance. Here, we propose a novel mechanism by which low-dose LPS inhalation in neonatal mice induces endotoxin tolerance, thereby offering protection from later asthma development. Three-day old wild-type and Toll-like receptor 4 (TLR4)-deficient neonatal mice were exposed to low-dose LPS (1 μg) intranasally for 10 consecutive days prior to ovalbumin (OVA)-induced asthma to better understand the tolerogenic mechanism(s) of low-dose LPS pre-exposure. *In vivo* findings were validated using *in vitro* co-culturing studies of primary $CD11c^+$ DCs and $CD4^+$ T-cells with or without low-dose LPS pre-exposure before OVA stimulation. Low-dose LPS pre-exposure upregulated the Treg response and downregulated pathogenic Th2 and Th17 responses through promoting apoptosis of Th2 and Th17 cells. Low-dose LPS pre-exposure downregulated DC GITRL expression and T-cell GITR expression. Artificial DC GITRL expression abrogated the tolerogenic Treg-skewing effect of low-dose LPS pre-exposure. Low-dose LPS pre-exposure inhibited TRIF/IRF3/IFNβ signaling and upregulated expression of tolerogenic TRIF/IRF3/IFNβ negative regulators in a TLR4-dependent manner. This tolerogenic DC GITRL downregulation was attributable to TRIF/IRF3/IFNβ signaling inhibition. Low-dose LPS pre-exposure produces tolerogenic Treg skewing in neonatal asthmatic mice, a phenomenon attributable to TLR4-dependent TRIF/IRF3/IFNβ-mediated DC GITRL downregulation.

Keywords: asthma, LPS, endotoxin tolerance, TLR4, GITRL

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Susetta Finotto,
University Hospital Erlangen, Germany
Takeshi Fujii,
Doshisha Women's College of Liberal Arts, Japan
Simona Ronchetti,
University of Perugia, Italy
Tian-Yong Hu,
Longgang ENT Hospital & Shenzhen ENT Institute, China

****Correspondence:***
Zhou Fu
Fuzhoucqmu@126.com

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *08 June 2020*
Accepted: *07 August 2020*
Published: *23 September 2020*

Citation:
Ding F, Liu B, Niu C, Wang T, Wang Y, Geng G, Tian D, Dai J and Fu Z (2020) Low-Dose LPS Induces Tolerogenic Treg Skewing in Asthma. Front. Immunol. 11:2150. doi: 10.3389/fimmu.2020.02150

Abbreviations: AHR, airway hyperresponsiveness; APCs, antigen-presenting cells; DCs, dendritic cells; GITRL, glucocorticoid-induced tumor necrosis factor receptor ligand; IFNβ, interferon-β; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; OVA, ovalbumin; TLR4, Toll-like receptor 4; Treg, regulatory T-cell; TRIF, TIR-domain-containing adapter-inducing interferon-β.

INTRODUCTION

Asthma is an allergen-derived immunologic disorder characterized by airway hyperresponsiveness (AHR), chronic airway inflammation, and enhanced allergen-specific IgE production (1). Although the incidence of asthma has been increasing globally (2), asthma medications are mainly anti-symptomatic, and allergen-specific immunotherapy does not show strong evidence of efficacy (3). Thus, blocking the effects of the immune response in the critical period before asthma formation has become an important issue for both researchers and clinicians.

Notably, epidemiological studies have demonstrated that children exposed to environments rich in the endotoxin lipopolysaccharide (LPS) have a lower prevalence of asthma and other allergic diseases, a phenomenon termed endotoxin tolerance (4, 5). For instance, environmental endotoxin exposure, such as from LPS present in dust from mattresses or kitchen floors, has been shown to protect against asthma and atopy in children residing in farming and non-farming households (4, 6). On this basis, LPS may be a valuable tool in inducing endotoxin tolerance prior to asthma development. However, a recent study has demonstrated that differing levels of LPS exposure can produce divergent immune responses in murine models of asthma (7). Therefore, the precise mechanism(s) underlying endotoxin tolerance remain unclear and require elucidation.

$CD4^+$ T-cells, comprised mainly of T-helper (Th)1, Th2, Th17, and regulatory T (Treg) cells, are critical cellular mediators of asthma (8). Although an impaired Th1/Th2 balance in favor of Th2 cells has been clearly established in asthma patients (9), the role of Treg cells has also gained interest among asthma researchers (10, 11). As Treg cells function in opposition to T-helper cells, their main function in asthma is to negatively regulate Th1, Th2, and Th17 cells to prevent Th-cell hyperactivity (10, 11). Accordingly, targeted depletion of Treg cells has been shown to aggravate a murine model of asthma (12), while adoptive transfer of induced Treg cells produces tolerogenic effects (13, 14). As early childhood exposure to LPS-expressing microorganisms induces Treg cells and suppresses aberrant Th2 immune responses (15), Treg-mediated Th cell suppression may play a critical role in the induction of endotoxin tolerance. However, the molecular mechanism(s) underlying this tolerogenic phenomenon remain unclear.

Dendritic cells (DCs) are allergen-sensing, antigen-presenting cells (APCs) that activate T-cells and direct their differentiation toward Th1, Th2, Th17, or Treg lineages (15). One key co-stimulatory molecule present on the surface of DCs—glucocorticoid-induced tumor necrosis factor receptor ligand (GITRL)—*via* binding to its corresponding T-cell ligand GITR serves to inhibit Treg-mediated Th cell suppression and enhance Th2 cell activity, thus augmenting AHR, serum IgE levels, and Th2 cytokine release in a murine model of asthma (16). As LPS has been shown to affect GITRL expression on DCs (17), we hypothesized that LPS-induced changes in DC GITRL expression may impact Treg-mediated Th cell suppression and the induction of endotoxin tolerance. In this study, we propose a novel mechanism by which low-dose LPS inhalation in neonatal mice induces endotoxin tolerance, thereby offering protection from later asthma development.

MATERIALS AND METHODS

The methods are fully detailed in the **Supplementary Material**.

RESULTS

Construction of Optimal LPS Pre-exposure Model

To explore the effect of LPS pre-exposure during immune maturation in early life, neonatal mice were pre-exposed to two different doses of LPS (1 or 100 μg) at two different time points after birth 3rd or 14th day of life (DOL) before the ovalbumin (OVA)-induced asthma model was established (**Supplementary Figure 1**). These various LPS pre-exposure protocols exhibited different effects on asthma development. Notably, newborn mice with the lower dose of LPS (3d1μgLPS/OVA) were significantly protected from asthma with significantly reduced AHR ($p < 0.05$, **Supplementary Figure 2A**), significantly reduced peribronchial and perivascular inflammation in lung tissues ($p < 0.05$, **Supplementary Figure 2B**), significantly reduced serum OVA-specific IgE levels ($p < 0.05$, **Supplementary Figure 2C**), and significantly reduced bronchoalveolar lavage fluid (BALF) levels of pro-eosinophilic/neutrophilic cytokines ($p < 0.05$, **Supplementary Figure 3C**). Thus, the 3d1μgLPS/OVA model was chosen as the optimal LPS pre-exposure model for subsequent experiments (**Figure 1**).

LPS Pre-exposure Promotes Treg Skewing *in vivo*

Although the exact mechanism(s) underlying endotoxin tolerance in asthma are still unclear, current evidence suggests that T-cell distributions favoring Treg ($CD4^+CD25^+Foxp3^+$) or Th1 cells (which are induced by endotoxin exposure) over pathogenic Th2 ($CD4^+IL\text{-}4^+$) and Th17 ($CD4^+IL\text{-}17^+$) cells, as well as the suppression of DCs and barrier epithelial cells, may play important roles in the development of endotoxin tolerance (18). Therefore, using the aforementioned 3d1μgLPS/OVA model, we assessed the changes of $CD4^+$ T-cell profiles between control, PBS/OVA, 3d1μgLPS/OVA, and 3d1μgLPS/PBS mice. After asthma induction, the proportion of Treg cells and Treg-associated Foxp3 mRNA expression significantly decreased, whereas the proportions of Th2 cells (and Th2-associated GATA3 mRNA expression) as well as Th17 cells (and Th17-associated ROR-γt mRNA expression) significantly increased in PBS/OVA mice relative to the control group ($p < 0.05$, **Supplementary Figures 3A–B**). Accordingly, asthma induction also significantly decreased Treg-associated IL-10 and TGF-β production while significantly increasing Th2-associated IL-4, IL-5, and IL-13 production and Th17-associated IL-17 production ($p < 0.05$, **Supplementary Figure 3C**). Consistent with our hypothesis, the 3d1μgLPS/OVA mice displayed significant reversal of these asthma-induced effects, with significant increases in the

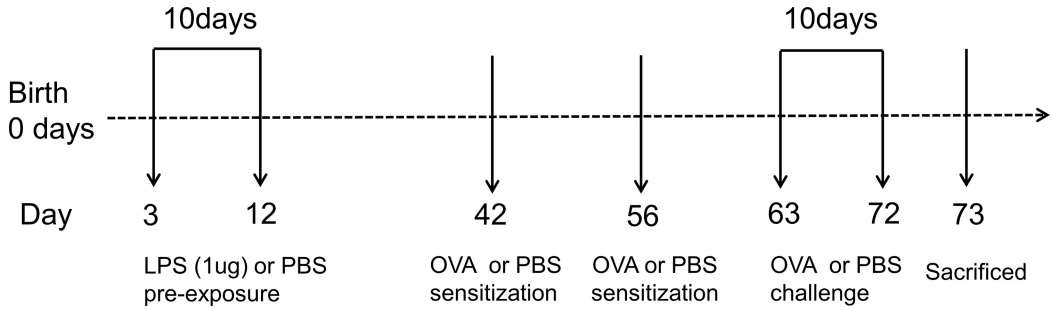


FIGURE 1 | Experimental protocol for constructing murine model of asthma. Neonatal BALB/c mice received daily low-dose LPS (1 μg) or PBS (control) intranasally (i.n.) from the 3rd DOL for a period of 10 consecutive days. Mice were then sensitized with 100 μg OVA plus 100 μl aluminum hydroxide (AlOH) or PBS (control) *via* intraperitoneal (i.p.) injection on the 42nd DOL and 56th DOL, and then further exposed to a daily, 30-min 1% OVA aerosol or PBS aerosol (control) from the 63rd DOL for a period of 10 consecutive days. Twenty-four hours after the last OVA challenge, the mice were sacrificed. n = 6–8 mice per group.

proportion of Treg cells (and their associated markers) and significant decreases in the proportions of Th2 and Th17 cells (and their associated markers) as compared to those of PBS/OVA mice ($p < 0.05$, **Supplementary Figures 3A–C**). However, simple exposure to 1 μg LPS without asthma induction (LPS/PBS) did not significantly affect Foxp3, GATA3, or ROR-γt mRNA levels nor IL-10, TGF-β, IL-4, IL-5, IL-13, or IL-17 levels ($p > 0.05$, **Supplementary Figures 3A–C**) compared to those of Control mice. These results demonstrate that pre-exposure with low-dose LPS upregulates the Treg response and downregulates Th2 and Th17 responses, thereby producing positive Treg skewing in neonatal asthmatic mice.

LPS Pre-exposure Increases Apoptosis of Th2 and Th17 Cells *in vitro*

Having shown that low-dose LPS pre-exposure produces Treg skewing *in vivo*, we next examined the effects of low-dose LPS pre-exposure on apoptosis levels of the $CD4^+$ T-cell subsets *in vitro*. We separated Treg, Th1, Th2, and Th17 cells by flow cytometry and respectively analyzed cleaved caspase-3 expression and apoptotic cell percentages in these subsets (**Supplementary Figure 4**). Cleaved caspase-3 expression and apoptotic cell percentages of Th2 and Th17 cells were significantly decreased under PBS/OVA conditions as compared to control conditions ($p < 0.05$, **Supplementary Figures 4C,D**). Notably, cleaved caspase-3 expression and apoptotic cell percentages of Th2 and Th17 cells increased significantly in LPS/OVA cells as compared with PBS/OVA cells ($p < 0.05$, **Supplementary Figures 4C,D**). However, no significant changes in cleaved caspase-3 expression or apoptotic cell percentages were observed in Treg cells or Th1 cells ($p > 0.05$, **Supplementary Figures 4A,B**). These results support our *in vivo* findings that pre-exposure with low-dose LPS exposure produces Treg skewing, likely through promoting apoptosis of pathogenic Th2 and Th17 cells.

LPS Pre-exposure Downregulates DC GITRL and T-Cell GITR Expression *in vivo*

Stimulation of Treg-expressed GITR by DC-expressed GITRL has been shown to abolish Treg suppression and increase the proliferation of effector T-cells resistant to Treg suppressive activity (16), and LPS has been shown to affect GITRL expression on DCs (17). We hypothesized that the rebalancing between Treg cells and effector T-cells observed after pre-exposure with low-dose LPS in asthmatic mice may be due to altered GITR and GITRL expression. Indeed, we found that GITRL expression in lung-derived DCs from 3d1μgLPS/OVA mice was significantly lower than that in PBS control mice according to immunohistochemistry ($p < 0.05$, **Figures 2A,B**), immunofluorescence ($p < 0.05$, **Figures 2C,D**), membrane-fraction western blotting ($p < 0.05$, **Figures 2E,F**), and flow cytometry (2.53 ± 0.46 vs. 6.82 ± 0.78 vs. 3.43 ± 0.74 vs. 2.55 ± 0.63, $p < 0.05$, **Figure 2G**).

We further studied GITR expression on Tregs as well as Th1, Th2, and Th17 cells extracted from mice. Mirroring GITRL expression on DCs, our results show that the levels of GITR on all T-cell subtypes tested in 3d1μgLPS/OVA mice were significantly lower than those in PBS/OVA mice ($p < 0.05$, **Supplementary Figure 5**). These results show that DC GITRL expression and T-cell GITR expression are downregulated *in vivo* by pre-exposure with low-dose LPS in our murine model of asthma.

LPS Pre-exposure Downregulates DC GITRL and T-Cell GITR Expression *in vitro*

In order to validate our *in vivo* findings, we further confirmed the effects of low-dose LPS pre-exposure on DC GITRL expression and T-cell GITR expression using *in vitro* co-culturing studies. Primary $CD11c^+CD11b^+$ DCs and $CD4^+$ T-cells were extracted and sorted for co-culture with or without low-dose LPS pre-exposure (100 ng/ml) before OVA peptide stimulation (1 μg/ml). Employing immunofluorescence, membrane-fraction Western blotting, and flow cytometry, we demonstrated that GITRL expression on PBS/OVA DCs was higher than control DCs ($p < 0.05$, **Figures 3A–C**), and expression of GITRL significantly decreased after pre-exposure with low-dose LPS (LPS/OVA) ($p < 0.05$, **Figures 3A–C**). In parallel, the levels of GITR on Treg, Th2, and Th17 cells in PBS/OVA mice were significantly higher than matching cells from control mice, whereas the GITR

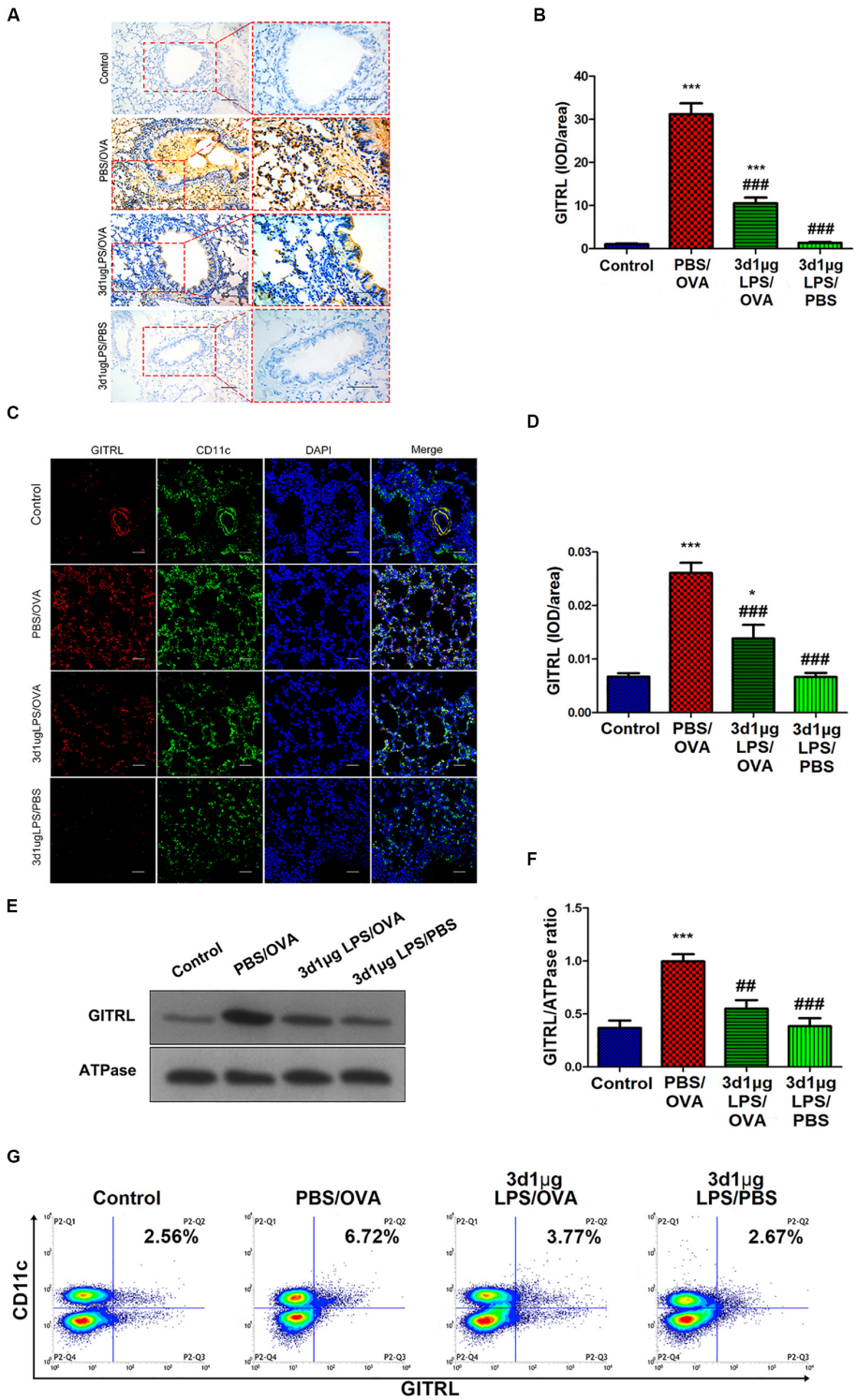


FIGURE 2 | Lung-derived dendritic cell GITRL expression downregulated in low-dose LPS-pre-exposed asthmatic mice relative to untreated asthmatic mice. **(A,B)** Immunohistochemistry of GITRL in lung tissue sections. Left panel (200× magnification) scale bars = 50 μm, right panel (400× magnification) scale bars = 100 μm. **(C,D)** Immunofluorescence of GITRL in lung tissue sections. Scale bars (200× magnification) = 50 μm. **(E,F)** Membrane-fraction immunoblotting of GITRL expression in lung-derived $CD11c^{+}CD11b^{+}$ dendritic cells. **(G)** Flow cytometry of surface GITRL expression on lung-derived $CD11c^{+}CD11b^{+}$ dendritic cells. n = 6–8 mice per group. Data are reported as means ± standard deviations (SDs). $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. Control group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. PBS/OVA group. Control, unexposed normal mice; PBS/OVA, asthmatic mice; 3d1μgLPS/OVA, low-dose (1 μg daily) LPS-exposed asthmatic mice with LPS exposure at 3rd DOL; 3d1μgLPS/PBS, low-dose (1 μg daily) LPS-exposed normal mice with LPS exposure at 3rd DOL.

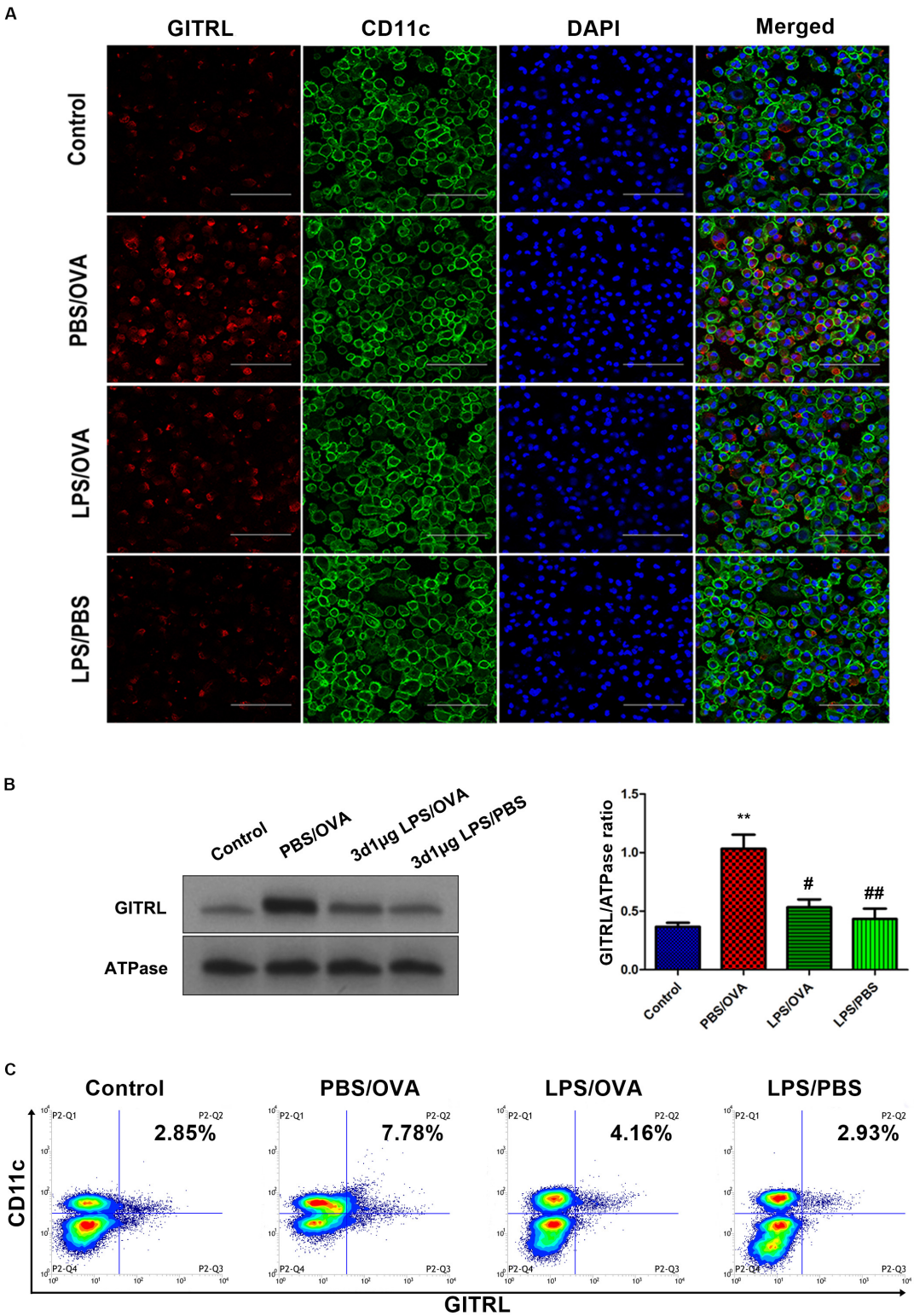


FIGURE 3 | Co-culture of low-dose LPS-pre-exposed dendritic cells and T-cells prior to OVA stimulation downregulates dendritic cell GITRL expression. GITRL expression on $CD11c^{+}CD11b^{+}$ dendritic cells were significantly downregulated in the low-dose LPS-exposed LPS/OVA cells **(**100 ng/ml LPS) as compared with PBS/OVA cells. **(A)** Surface GITRL expression on primary dendritic cells by immunofluorescence. Scale bars (600× magnification) = 150 μm. **(B)** Membrane-fraction immunoblotting of GITRL expression on primary dendritic cells. **(C)** Surface GITRL expression on primary dendritic cells by flow cytometry. Data are reported as means ± standard deviations (SDs). $^{**}p < 0.01$ vs. Control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. PBS/OVA group. Control, unexposed normal mice; PBS/OVA, asthmatic mice; LPS/OVA, low-dose LPS-exposed asthmatic mice; LPS/PBS, low-dose LPS-exposed normal mice.

expression was significantly decreased after pre-exposure with low-dose LPS (LPS/OVA) (**Supplementary Figure 6**, $p < 0.05$). Notably, GITR expression on Th1 cells was not significantly different between the experimental groups (**Supplementary Figure 6B**, $p > 0.05$). These *in vitro* results confirm that DC GITRL expression and T-cell GITR expression are downregulated by pre-exposure to low-dose LPS.

Artificial Overexpression of DC GITRL Abrogates the Tolerogenic Treg-Skewing Effect of Low-Dose LPS Pre-exposure

To determine whether the effects of low-dose LPS pre-exposure are GITRL-dependent, we next altered bone-marrow-derived DC GITRL expression by transfection with either a GITRL siRNA or a recombinant pEGFP-N1-GITRL overexpression plasmid and then adoptively transferred these transfected DCs into mice. To validate that the DCs successfully transferred and migrated to the lung, we confirmed stable GITRL transcript knockdown and overexpression in lung-derived DCs from GITRL-silenced 3d1μgLPS/OVA and GITRL-overexpressing 3d1μgLPS/OVA mice, respectively ($p < 0.05$, **Supplementary Figure 7**).

glucocorticoid-induced tumor necrosis factor receptor ligand-overexpressing 3d1μgLPS/OVA mice showed significantly higher peribronchial and perivascular inflammation in lung tissues ($p < 0.05$, **Figure 4A**), significantly higher levels of AHR ($p < 0.05$, **Figure 4B**), significantly higher inflammation score ($p < 0.05$, **Figure 4C**), significantly higher serum OVA-specific IgE levels ($p < 0.05$, **Figure 4D**) and significantly higher BALF levels of pro-eosinophilic/neutrophilic cytokines ($p < 0.05$, **Supplementary Figure 8C**) as compared to 3d1μgLPS/OVA mice. Moreover, GITRL-overexpressing 3d1μgLPS/OVA mice displayed significant decreases in Treg levels and Treg-associated Foxp3 mRNA expression accompanied by significant increases in Th2 levels and Th2-associated GATA3 mRNA expression as well as significant increases in Th17 levels and Th17-associated ROR-γt mRNA expression as compared to 3d1μgLPS/OVA mice ($p < 0.05$, **Supplementary Figures 8A,B**). Changes in BALF cytokine levels paralleled the changes in T-cell subset composition ($p < 0.05$, **Supplementary Figure 8C**). The opposite effects were observed in GITRL-silenced 3d1μgLPS/OVA mice ($p < 0.05$, **Figures 4A–C** and **Supplementary Figures 8A–C**).

In order to validate our *in vivo* findings, we further confirmed the effects of altered GITRL expression using *in vitro* co-culturing studies. Primary transfected $CD11c^+CD11b^+$ DCs and $CD4^+$ T-cells were co-cultured with or without low-dose LPS pre-exposure before OVA stimulation. We first validated stable GITRL transcript knockdown and overexpression in these primary transfected DCs ($p < 0.05$, **Supplementary Figure 9**). Similar to our *in vivo* findings, GITRL-overexpressing LPS/OVA DCs produced significant decreases in Treg levels and Treg-associated Foxp3 mRNA expression accompanied by significant increases in Th2 levels and Th2-associated GATA3 mRNA expression as well as significant increases in Th17 levels and Th17-associated ROR-γt mRNA expression as compared to LPS/OVA DCs ($p < 0.05$, **Supplementary Figures 10A,B**). Changes in BALF cytokine levels paralleled the changes in T-cell subset composition ($p < 0.05$, **Supplementary Figure 10C**). The opposite effects were observed with GITRL-silenced LPS/OVA DCs ($p < 0.05$, **Supplementary Figures 10A–C**). These combined results demonstrate that the tolerogenic Treg-skewing effect of low-dose LPS pre-exposure is abrogated by artificial DC GITRL expression.

LPS Pre-exposure Downregulates DC TRIF/IRF3/IFNβ Pathway Activation in a TLR4-Dependent Manner

Having shown that the tolerogenic effects of low-dose LPS pre-exposure are due to GITRL downregulation on DCs, we next examined the molecular mechanism(s) underlying DC GITRL downregulation following low-dose LPS pre-exposure. LPS binds to Toll-like receptor 4 (TLR4) expressed on the surface of APCs, and this LPS-TLR4 ligation regulates the downstream TRIF/IRF3/IFNβ (TIR-domain-containing adapter-inducing interferon-β/interferon regulatory factor 3/interferon-β) pathway *via* multiple negative feedback loops (19, 20). Given the fact that IFNβ stimulates GITRL expression (21), we hypothesized that TLR4-dependent TRIF/IRF3/IFNβ signaling inhibition is responsible for the GITRL downregulation observed in low-dose LPS pre-exposed DCs.

To determine whether the effects of low-dose LPS pre-exposure are TLR4-dependent, we next obtained TLR4-deficient $CD11c^+CD11b^+$ DCs from the bone marrow of TLR4 knockout (KO) mice. Then, either wild-type (WT) DCs or TLR4-KO DCs were co-cultured with $CD4^+$ T-cells with or without low-dose LPS pre-exposure before OVA stimulation in order to determine the downstream effects of TLR4. We first validated stable TLR4 expression and stable TLR4 knockdown in the WT and TLR4-KO DCs, respectively, *via* immunoprecipitation with TRIF and simple immunoblotting ($p < 0.05$, **Figures 5A,B**). PBS/OVA WT DCs showed significantly higher activation of the downstream TRIF/IRF3/IFNβ signaling cascade compared to control DCs ($p < 0.05$, **Figures 5B,C**). Notably, TRIF/IRF3/IFNβ signaling was significantly decreased in WT DCs after pre-exposure with low-dose LPS (LPS/OVA) ($p < 0.05$, **Figures 5B,C**). In contrast, this inhibitory effect of low-dose LPS pre-exposure on TRIF/IRF3/IFNβ signaling was completely abrogated in TLR4-KO DCs ($p > 0.05$, **Figures 5B,C**). These results demonstrate that the inhibitory effect of low-dose LPS pre-exposure on downstream TRIF/IRF3/IFNβ signaling in DCs is TLR4-dependent.

In order to further investigate the mechanism(s) by which low-dose LPS pre-exposure affects TRIF/IRF3/IFNβ signaling in DCs, we next measured the expression of several TLR4-associated negative regulators of TRIF/IRF3/IFNβ signaling, including SARM (Sterile alpha and armadillo-motif containing protein), PTPB1 (Polypyrimidine-tract binding protein), SIKE (Suppressor of IKK epsilon), and SHP2 (Src homology region 2-containing protein tyrosine phosphatase 2) (22–24). PBS/OVA WT DCs showed significant downregulation of SARM, PTPB1, and SIKE compared to control DCs ($p < 0.05$, **Figure 5D**). Notably, LPS/OVA WT DCs displayed significant upregulation of these negative regulators compared to PBS/OVA WT DCs

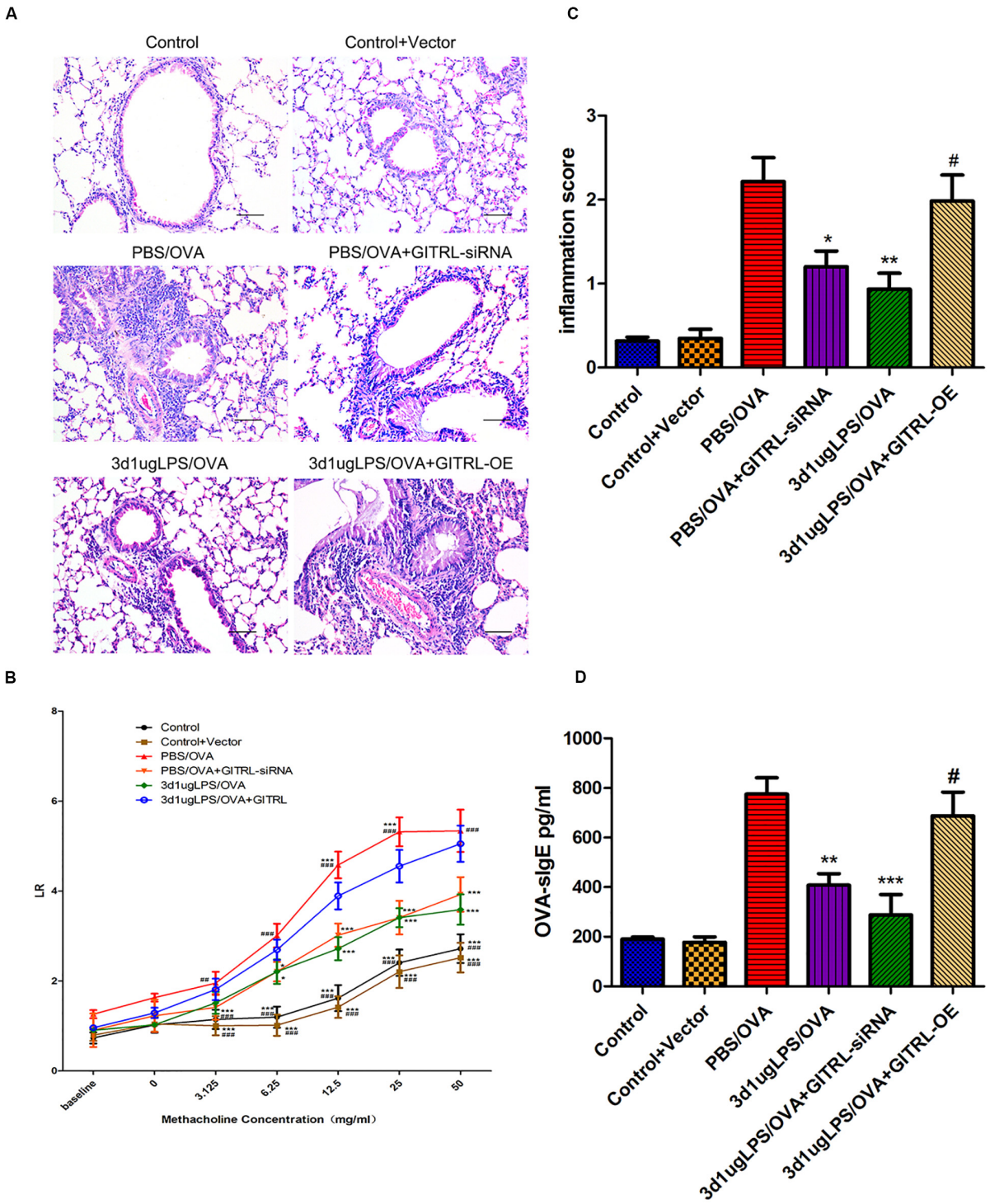


FIGURE 4 | Pulmonary effects of GITRL silencing or overexpression on low-dose LPS-pre-exposed asthmatic mice. **(A)** Representative images of lung tissue sections stained with hematoxylin and eosin (H&E) 24 h after the final challenge. Left panel (200× magnification) scale bars = 50 μm, right panel (400× magnification) scale bars = 100 μm. **(B)** Lung resistance (LR) values in reaction to increasing doses of methacholine were measured 24 h after the final challenge. **(C)** Inflammation score in each group. **(D)** Serum OVA-specific IgE levels as measured by ELISA. n = 6–8 mice per group. Data are reported as means ± standard deviations (SDs). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. PBS/OVA group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. 3d1μgLPS/OVA group. Control, unexposed normal mice; Control + Vector, unexposed normal mice with empty vector; PBS/OVA, asthmatic mice; 3d1μgLPS/OVA, low-dose LPS-exposed asthmatic mice; 3d1μgLPS/OVA + GITRL-siRNA, low dose LPS-exposed asthmatic mice with GITRL-siRNA DCs; 3d1μgLPS/OVA + GITRL-OE, low dose LPS-exposed asthmatic mice with GITRL-overexpressing DCs.

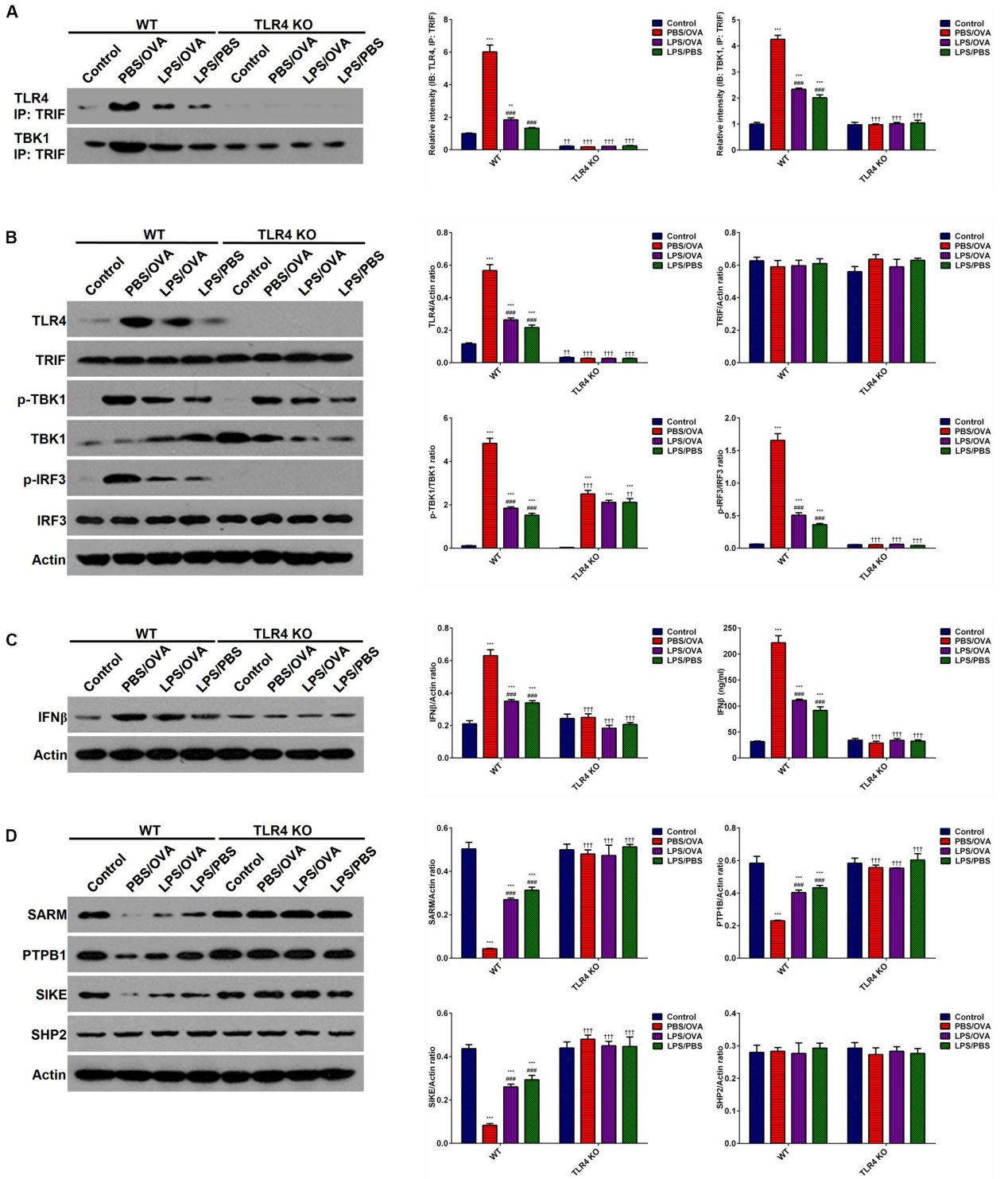


FIGURE 5 | Low-dose LPS pre-exposure downregulates TLR4-dependent TRIF/IRF3/IFNβ pathway activation in dendritic cells. TLR4-mediated TRIF/IRF3/IFNβ pathway activation in $CD11c^{+}CD11b^{+}$ dendritic cells was significantly downregulated in the low-dose LPS-exposed LPS/OVA cells **(**100 ng/ml LPS) as compared with PBS/OVA cells. **(A)** Whole-cell lysate immunoblotting of TLR4 and TBK1 following immunoprecipitation with anti-TRIF antibodies in primary dendritic cells. **(B)** Whole-cell lysate immunoblotting of TLR4/TRIF/TBK1/IRF3 pathway proteins in primary dendritic cells. **(C)** Whole-cell lysate immunoblotting of IFNβ (left panels) and supernatant levels of IFNβ as measured by ELISA (right panel) in primary dendritic cells. **(D)** Whole-cell lysate immunoblotting of four key negative regulators of the TLR4/TRIF/TBK1/IRF3 pathway proteins in primary dendritic cells. Data are reported as means ± standard deviations (SDs). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Control group; $^{\#\#\#}p < 0.001$ vs. PBS/OVA group; $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ vs. WT.

($p < 0.05$, **Figure 5D**). In contrast, this upregulating effect of low-dose LPS pre-exposure on SARM, PTPB1, and SIKE expression was completely abrogated in TLR4-KO DCs ($p > 0.05$, **Figure 5D**). These results suggest that the inhibitory effect of low-dose LPS pre-exposure on TRIF/IRF3/IFNβ signaling in DCs may be mediated by the negative regulators SARM, PTPB1, and SIKE in a TLR4-dependent manner.

In order to further validate our findings, we took a deeper look at IRF3. It is well-established that TBK1-phosphorylated IRF3 dimerizes in the cytoplasm and translocates to the nucleus, where it transactivates several potent genes, most notably IFNβ (22). Here, we validated that PBS/OVA WT DCs displayed significantly higher IRF3 dimerization, nuclear translocation, and transactivation potential compared to control DCs ($p < 0.05$, **Supplementary Figures 11A–C**). Notably, these effects were significantly decreased in WT DCs after pre-exposure with low-dose LPS (LPS/OVA) ($p < 0.05$, **Supplementary Figures 11A–C**). In contrast, these inhibitory effects of low-dose LPS pre-exposure on IRF3 activation were completely abrogated in TLR4-KO DCs ($p > 0.05$, **Supplementary Figures 11A–C**).

IFNβ Exposure Rescues Dendritic Cell GITRL Downregulation From Low-Dose LPS Pre-exposure

Having demonstrated the inhibitory effects of low-dose LPS pre-exposure on TRIF/IRF3/IFNβ signaling in DCs and given the fact that IFNβ stimulates GITRL expression (21), we surmised that exogenous IFNβ exposure should rescue GITRL downregulation in low-dose LPS pre-exposed DCs. *In vitro*, primary $CD11c^+CD11b^+$ DCs and $CD4^+$ T-cells were co-cultured with or without low-dose LPS pre-exposure in the presence of absence of IFNβ prior to OVA stimulation. By both flow cytometry and membrane-fraction immunoblotting, we found that IFNβ exposure completely rescued GITRL downregulation in low-dose LPS pre-exposed DCs ($p < 0.05$, **Figures 6A,B**).

In order to validate these findings *in vivo*, we employed the aforedescribed 3d1μgLPS/OVA murine model (both with and without IFNβ exposure during the LPS pre-exposure period) and assessed the changes in DC GITRL expression between control, PBS/OVA, 3d1μgLPS/OVA, and 3d1μgLPS/PBS mice. Consistent with our *in vitro* results, we found that IFNβ exposure completely rescued GITRL downregulation in 3d1μgLPS/OVA DCs ($p < 0.05$, **Figures 6C,D**). These combined findings reveal that the GITRL downregulation on DCs from low-dose LPS pre-exposure is attributable to decreased TRIF/IRF3/IFNβ signaling.

DISCUSSION

In this study, we hypothesized that LPS-induced changes in DC GITRL expression may impact Treg-mediated Th cell suppression and the induction of endotoxin tolerance. We found that low-dose LPS pre-exposure (1 μg) in neonatal asthmatic mice produces Treg skewing *via* promoting apoptosis of pathogenic Th2 and Th17 cells through downregulating DC GITRL expression. We also demonstrated that this DC GITRL downregulation is attributable to TLR4-dependent TRIF/IRF3/IFNβ signaling inhibition. These combined findings reveal that low-dose LPS pre-exposure produces tolerogenic Treg skewing in neonatal asthmatic mice, a phenomenon attributable to TLR4-dependent TRIF/IRF3/IFNβ-mediated DC GITRL downregulation.

In humans, the majority of asthma cases debut before the age of three with loss of lung function evident by 6 years of age (25). During these early years, environmental LPS exposure appears to play a critical role in either endotoxin tolerance or asthma development (4–6). Moreover, differing levels of LPS exposure can produce divergent immune responses in murine models of asthma (7). In order to construct an optimal murine model of endotoxin tolerance, here we applied various concentrations of LPS at different time points during the neonatal immune maturation period prior to OVA-induced asthma sensitization and challenge. We observed that low-dose (1 μg) LPS inhalation in 3-day old neonatal mice reduced AHR, peribronchial, and perivascular inflammation in lung tissues, serum OVA-specific IgE levels, and BALF levels of pro-eosinophilic/neutrophilic cytokines. On this basis, low-dose LPS pre-exposure in neonates appears to have a tolerogenic effect on later asthma development. Moreover, our findings suggest that variations in the exposure level and timing of environmental LPS exposure may explain the discordant phenotypes following LPS exposure.

Although the exact mechanism(s) underlying endotoxin tolerance in asthma remain unclear, current evidence suggests that T-cell distributions favoring Treg or Th1 cells over pathogenic Th2 and Th17 cells play important roles in the development of endotoxin tolerance (18). This is because Th2 cells produce the type 2 cytokines IL-4, IL-5, and IL-13 that drive eosinophilic inflammation and mucus production (26), while Th17 cell-produced IL-17 induces pathogenic smooth muscle contractions and structural alterations to the airway epithelium (27). Here, from both *in vivo* and *in vitro* experimentation, we found that low-dose LPS pre-exposure produces Treg skewing by promoting apoptosis of pathogenic Th2 and Th17 cells. Our findings are consistent with previous research demonstrating that Treg and/or Th1 skewing confers tolerogenic effects against asthma and other allergic disorders (28, 29).

The DC-expressed co-stimulatory molecule GITRL plays an important role in inhibiting Treg-mediated suppression of Th cells, thereby inhibiting tolerogenicity and eliciting autoimmune disease (16). Here, from both *in vivo* and *in vitro* experimentation, we found that low-dose LPS pre-exposure produces Treg skewing by promoting apoptosis of pathogenic Th2 and Th17 cells through downregulating DC GITRL expression. To confirm that GITRL downregulation was responsible for LPS's tolerogenic effects, we silenced and overexpressed GITRL in lung-derived murine DCs and adoptively transferred these transfected DCs into mice. The adoptive transfer of GITRL-overexpressing DCs abrogated the tolerogenic effects of LPS pre-exposure as evidenced by increases in AHR, peribronchial, and perivascular inflammation in lung tissue, serum OVA-specific IgE levels, and BALF levels of pro-eosinophilic/neutrophilic cytokines. In contrast, the adoptive transfer of GITRL-silenced DCs produced the opposite effects.

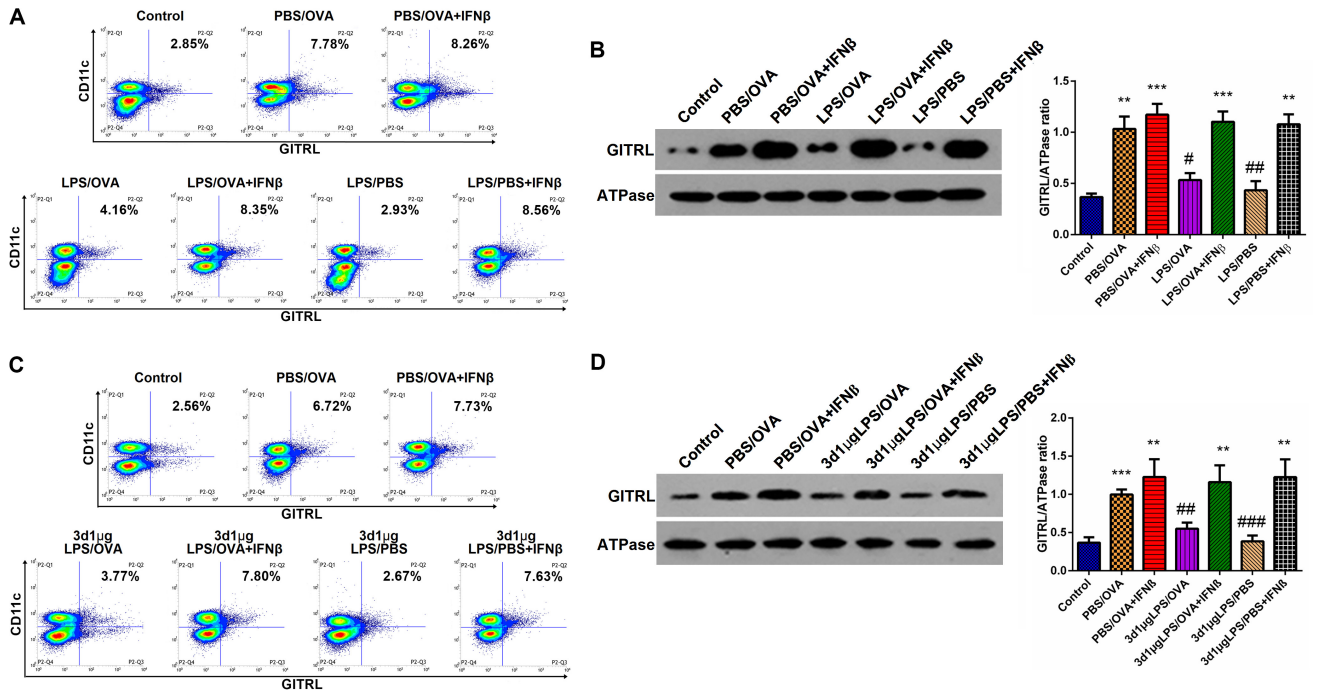


FIGURE 6 | IFNβ exposure rescues dendritic cell GITRL downregulation from low-dose LPS pre-exposure. **(A)** Surface GITRL expression on primary $CD11c^+CD11b^+$ dendritic cells by flow cytometry. **(B)** Membrane-fraction immunoblotting of GITRL expression on primary $CD11c^+CD11b^+$ dendritic cells. **(C)** Flow cytometry of surface GITRL expression on lung-derived $CD11c^+CD11b^+$ dendritic cells. **(D)** Membrane-fraction immunoblotting of GITRL expression in lung-derived $CD11c^+CD11b^+$ dendritic cells. Data are reported as means ± standard deviations (SDs). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. PBS/OVA group. Control, unexposed normal mice; PBS/OVA, asthmatic mice; 3d1μgLPS/OVA, low-dose (1 μg daily) LPS-exposed asthmatic mice with LPS exposure at 3rd DOL; 3d1μgLPS/PBS, low-dose (1 μg daily) LPS-exposed normal mice with LPS exposure at 3rd DOL.

We validated these findings *in vitro via* co-culturing primary DCs with $CD4^+$ T-cells. It is important to note that GITRL overexpression in adoptively transferred DCs did not completely inhibit Treg-mediated suppression of Th cells. This might reflect the activity of APCs other than DCs.

We next examined the molecular mechanism(s) underlying DC GITRL downregulation following low-dose LPS pre-exposure. Interestingly, although LPS acts through the Toll-like receptor TLR4 expressed on the surface of APCs, LPS pre-exposure has divergent downstream effects depending on the dosage of the initial LPS challenge (19, 20). While pre-exposure to low or high doses of LPS can induce a transient pro-inflammatory state followed by a refractory tolerant state (endotoxin tolerance), pre-exposure to super-low doses of LPS (picogram levels) produces a non-resolving inflammatory adaptation, a phenomenon Morris et al. terms endotoxin priming (19, 20). Current evidence suggests that the "switch" between endotoxin tolerance vs. endotoxin priming results from a complex competition between two TLR4-mediated signaling pathways in APCs, namely the MyD88-dependent IRAK/MAPK pathway and the MyD88-independent TRIF/IRF3 pathway (20). Specifically, while the MyD88-dependent IRAK/MAPK pathway induces pro-inflammatory NF-κB activation, it also activates multiple tolerogenic negative feedback loops (20). In contrast, the MyD88-independent TRIF/IRF3 pathway prevents endotoxin tolerance by downregulating the expression of tolerogenic negative regulators, such as SIKE and SARM (20, 22). Therefore, there is a competitive dynamic balance between the MyD88-dependent IRAK/MAPK pathway (favoring endotoxin tolerance) vs. the MyD88-independent TRIF/IRF3 pathway (favoring endotoxin priming) (20). Consistent with this molecular model, here we demonstrated that low-dose LPS pre-exposure inhibited TRIF/IRF3/IFNβ signaling and upregulated expression of the tolerogenic negative regulators SARM, PTPB1, and SIKE. Mechanistically, as SARM and PTPB1 interfere with TLR4-TRIF binding and SIKE suppresses TBK1 activation, SARM, PTPB1, and SIKE upregulation would synergistically act to inhibit TRIF/IRF3/IFNβ signal transduction (22, 30). More importantly, the observed tolerogenic DC GITRL downregulation is also attributable to this inhibition in TRIF/IRF3/IFNβ signaling, as IFNβ exposure completely abolished GITRL downregulation both *in vitro* and *in vivo*.

In conclusion, low-dose LPS pre-exposure (1 μg) produces tolerogenic Treg skewing in neonatal asthmatic mice, a phenomenon attributable to TLR4-dependent TRIF/IRF3/IFNβ-mediated DC GITRL downregulation. Our findings provide important cellular and molecular insights into the criticality of LPS exposure levels and timing in the development of endotoxin tolerance, which help explain the discordant conclusions regarding the effects of early environmental endotoxin exposure on later allergic responses. Moreover, our findings may provide guidance on the development of novel preventative approaches

against asthma and other allergic disorders in young children *via* targeting the GITRL/GITR axis.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Research Advisory Committee at Chongqing Medical University.

AUTHOR CONTRIBUTIONS

ZF, FD, BL conceived and designed the study. FD, BL, CN, TW, and YW performed the experiments. YW, DT, GG, and JD analyzed the data. FD and ZF drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant No. 81800032).

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Caihui Gong for her comprehensive support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02150/full#supplementary-material

REFERENCES

1. FosterPS, MaltbyS, RosenbergHF, TayHL, HoganSP, CollisonAM, et al. Modeling th2 responses and airway inflammation to understand fundamental mechanisms regulating the pathogenesis of asthma. *ImmunolRev*. (2017) 278:20–40. doi: 10.1111/imr.12549
2. FerkolT, SchraufnagelD. The global burden of respiratory disease. *AnnAmThoracSoc*. (2014) 11:404–6. doi: 10.1513/annalsats.201311-405ps
3. Di BonaD, PlaiaA, Leto-BaroneM, La PianaS, MacchiaL, Di LorenzoG. Efficacy of allergen immunotherapy in reducing the likelihood of developing new allergen sensitizations: a systematic review. *Allergy*. (2017) 5:691–704. doi: 10.1111/all.13104
4. RiedlerJ, Braun-FahrländerC, EderW, SchreuerM, WaserM, MaischS, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet*. (2001) 358:1129–33. doi: 10.1016/s0140-6736(01)06252-3
5. Braun-FahrländerC, RiedlerJ, HerzU, EderW, WaserM, GrizeL, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N EnglJMed*. (2002) 347:869–77. doi: 10.1056/nejmoa020057
6. Von MutiusE, Braun-FahrlanderC, SchierlR, RiedlerJ, EhlermannS, MaischS, et al. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *ClinExpAllergy*. (2000) 30:1230–4. doi: 10.1046/j.1365-2222.2000.00959.x
7. KumarS, AdhikariA. Dose-dependent immunomodulating effects of endotoxin in allergic airway inflammation. *Innate Immun*. (2017) 23:249–57. doi: 10.1177/1753425917690443
8. Lovinsky-DesirS, MillerRL. Epigenetics, asthma, and allergic diseases: a review of the latest advancements. *CurrAllergyAsthma Rep*. (2012) 12:211–20. doi: 10.1007/s11882-012-0257-4
9. KiddP. Th1/th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern MedRev*. (2003) 8:223–46.
10. MamessierE, BotturiK, VervloetD, MagnanA. T regulatory lymphocytes, atopy and asthma: a new concept in three dimensions. *RevMalRespir*. (2005) 22(2 Pt 1):305–11. doi: 10.1016/S0761-8425(05)85482-8
11. PalomaresO, YamanG, AzkurAK, AkkocT, AkdisM, AkdisCA. Role of treg in immune regulation of allergic diseases. *EurJImmunol*. (2010) 40:1232–40. doi: 10.1002/eji.200940045
12. MaaziH, ShirinbakS, WillartM, HammadH, CabanskiM, BoonL, et al. Contribution of regulatory t cells to alleviation of experimental allergic asthma after specific immunotherapy. *ClinExpAllergy*. (2012) 42:1519–28. doi: 10.1111/j.1365-2222.2012.04064.x
13. XuW, LanQ, ChenM, ChenH, ZhuN, ZhouX, et al. Adoptive transfer of induced-treg cells effectively attenuates murine airway allergic inflammation. *PLoS One*. (2012) 7:e40314. doi: 10.1371/journal.pone.0040314
14. McGeeHS, AgrawalDK. Naturally occurring and inducible t-regulatory cells modulating immune response in allergic asthma. *AmJRespirCritCare Med*. (2009) 180:211–25. doi: 10.1164/rccm.200809-1505oc
15. KuoC-H, KuoH-F, HuangC-H, YangS-N, LeeM-S, HungC-H. Early life exposure to antibiotics and the risk of childhood allergic diseases: an update from the perspective of the hygiene hypothesis. *JMicrobiolImmunolInfect*. (2013) 46:320–9. doi: 10.1016/j.jmii.2013.04.005
16. MottaAC, VissersJL, GrasR, Van EschBC, Van OosterhoutAJ, NawijnMC. Gitr signaling potentiates airway hyperresponsiveness by enhancing th2 cell activity in a mouse model of asthma. *RespirRes*. (2009) 10:93. doi: 10.1186/1465-9921-10-93
17. YuK-Y, KimHS, SongSY, MinS-S, JeongJJ, YounB-S. Identification of a ligand for glucocorticoid-induced tumor necrosis factor receptor constitutively expressed in dendritic cells. *BiochemBiophysResCommun*. (2003) 310:433–8. doi: 10.1016/j.bbrc.2003.09.024
18. SchuijsMJ, WillartMA, VergoteK, GrasD, DeswarteK, EgeMJ, et al. Farm dust and endotoxin protect against allergy through a20 induction in lung epithelial cells. *Science*. (2015) 349:1106–10. doi: 10.1126/science.aac6623
19. MorrisMC, GilliamEA, LiL. Innate immune programing by endotoxin and its pathological consequences. *FrontImmunol*. (2014) 5:680. doi: 10.3389/fimmu.2014.00680
20. YuanR, GengS, LiL. Molecular mechanisms that underlie the dynamic adaptation of innate monocyte memory to varying stimulant strength of tlr ligands. *FrontImmunol*. (2016) 7:497. doi: 10.3389/fimmu.2016.00497
21. NocentiniG, RiccardiC. Gitr: a modulator of immune response and inflammation. *Adv Exp Med Biol*. (2009) 647:156–73. doi: 10.1007/978-0-387-89520-8_11
22. PiaoW, SongC, ChenH, DiazMAQ, WahlLM, FitzgeraldKA, et al. Endotoxin tolerance dysregulates myd88-and toll/il-1r domain-containing adapter inducing ifn-β-dependent pathways and increases expression of negative regulators of tlr signaling. *JLeukocBiol*. (2009) 86:863–75. doi: 10.1189/jlb.0309189
23. XuH, AnH, HouJ, HanC, WangP, YuY, et al. Phosphatase ptp1b negatively regulates myd88-and trif-dependent proinflammatory cytokine and type i interferon production in tlr-triggered macrophages. *MolImmunol*. (2008) 45:3545–52. doi: 10.1016/j.molimm.2008.05.006

24. AnH, ZhaoW, HouJ, ZhangY, XieY, ZhengY, et al. Shp-2 phosphatase negatively regulates the trif adaptor protein-dependent type i interferon and proinflammatory cytokine production. *Immunity*. (2006) 25:919–28. doi: 10.1016/j.immuni.2006.10.014
25. PanettieriRA, CovarR, GrantE, HillyerEV, BacharierL. Natural history of asthma: persistence versus progression—does the beginning predict the end? *JAllergyClinImmunol*. (2008) 121:607–13. doi: 10.1016/j.jaci.2008.01.006
26. BarlowJL, PeelS, FoxJ, PanovaV, HardmanCS, CameloA, et al. Il-33 is more potent than il-25 in provoking il-13–producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *JAllergyClinImmunol*. (2013) 132:933–41. doi: 10.1016/j.jaci.2013.05.012
27. ChesnéJ, BrazaF, MahayG, BrouardS, AronicaM, MagnanA. Il-17 in severe asthma. Where do we stand? *AmJRespirCritCare Med*. (2014) 190:1094–101. doi: 10.1164/rccm.201405-0859pp
28. RobinsonD. Regulatory T cells and asthma. *ClinExpAllergy*. (2009) 39:1314–23. doi: 10.1111/j.1365-2222.2009.03301.x
29. HamidQ, TulicM. Immunobiology of asthma. *AnnuRevPhysiol*. (2009) 71:489–507. doi: 10.1146/annurev.physiol.010908.163200
30. KawaiT, AkiraS. Snapshot: pattern-recognition receptors. *Cell*. (2007) 129:1024. doi: 10.1016/j.cell.2007.05.017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 29 September 2020
doi: 10.3389/fimmu.2020.02194

Induction of Antigen-Specific Tolerance in T Cell Mediated Diseases

*Laura Passerini and Silvia Gregori**

Mechanisms of Peripheral Tolerance Unit, San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy

The development of novel approaches to control unwanted immune responses represents an ambitious goal in the management of a number of clinical conditions, including autoimmunity, autoinflammatory diseases, allergies and replacement therapies, in which the T cell response to self or non-harmful antigens threatens the physiological function of tissues and organs. Current treatments for these conditions rely on the use of non-specific immunosuppressive agents and supportive therapies, which may efficiently dampen inflammation and compensate for organ dysfunction, but they require lifelong treatments not devoid of side effects. These limitations induced researchers to undertake the development of definitive and specific solutions to these disorders: the underlying principle of the novel approaches relies on the idea that empowering the tolerogenic arm of the immune system would restore the immune homeostasis and control the disease. Researchers effort resulted in the development of cell-free strategies, including gene vaccination, protein-based approaches and nanoparticles, and an increasing number of clinical trials tested the ability of adoptive transfer of regulatory cells, including T and myeloid cells. Here we will provide an overview of the most promising approaches currently under development, and we will discuss their potential advantages and limitations. The field is teaching us that the success of these strategies depends primarily on our ability to dampen antigen-specific responses without impairing protective immunity, and to manipulate directly or indirectly the immunomodulatory properties of antigen presenting cells, the ultimate *in vivo* mediators of tolerance.

Keywords: tolerance, dendritic cells, autoimmunity, cell therapy, immunomodulation, antigen-specific

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Jan Nilsson,
Lund University, Sweden
Barbara M. P. Willekens,
Antwerp University Hospital, Belgium
Carolin Daniel,
Helmholtz Zentrum München, Germany
David Markusic,
Indiana University, Purdue University Indianapolis, United States

***Correspondence:**
Silvia Gregori
gregori.silvia@hsr.it

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *11 June 2020*
Accepted: *11 August 2020*
Published: *29 September 2020*

Citation:
Passerini L and Gregori S (2020) Induction of Antigen-Specific Tolerance in T Cell Mediated Diseases. Front. Immunol. 11:2194. doi: 10.3389/fimmu.2020.02194

INTRODUCTION

The identification of novel approaches designed to selectively control antigen(Ag)-specific effector T (Teff) cell responses and promote or restore tolerance in T cell mediated diseases is an unsolved issue in the management of autoimmune diseases in humans. On this line, a new version of vaccination, also called "inverse vaccination," aims at inducing or restoring an immunological state of unresponsiveness, either toward foreign Ags (e.g., protein therapeutics, allergens, or transgenes) or autoAgs (1). The overall goal of inverse vaccination strategies is to dampen the adverse response, through deletion, inhibition or deviation of Ag-specific Teff cells, and to support the induction and/or expansion of Ag-specific T regulatory cells (Tregs). Tregs are recognized as a cell population responsible for induction and maintenance of immune tolerance. The best characterized subsets

are the Forkhead box P3 expressing Tregs (FOXP3$^+$ Tregs) (2) and the IL-10-producing type 1 regulatory (Tr1) cells (3).

A number of different strategies have been proposed as inverse vaccination: (i) cell-free based approaches, including gene vaccination and protein or peptide delivery; (ii) vehicle approaches, to deliver Ags by means of apoptotic cells, liposomes, or nanoparticles; (iii) cell-based approaches, aimed at providing specialized cells to reinforce the regulatory arm of the immune system. This Review aims to provide an overview of the most promising approaches currently under development and clinical testing (**Figure 1** and **Table 1**) and their potential advantages and limitations.

CELL FREE STRATEGIES

Inverse gene vaccination strategies aim at the induction of tolerance to a relevant Ag by means of transient expression of whole proteins or epitopes from DNA or RNA vectors in the absence of pro-inflammatory stimuli. Once injected, the coding sequence needs to enter the cytoplasm of the target

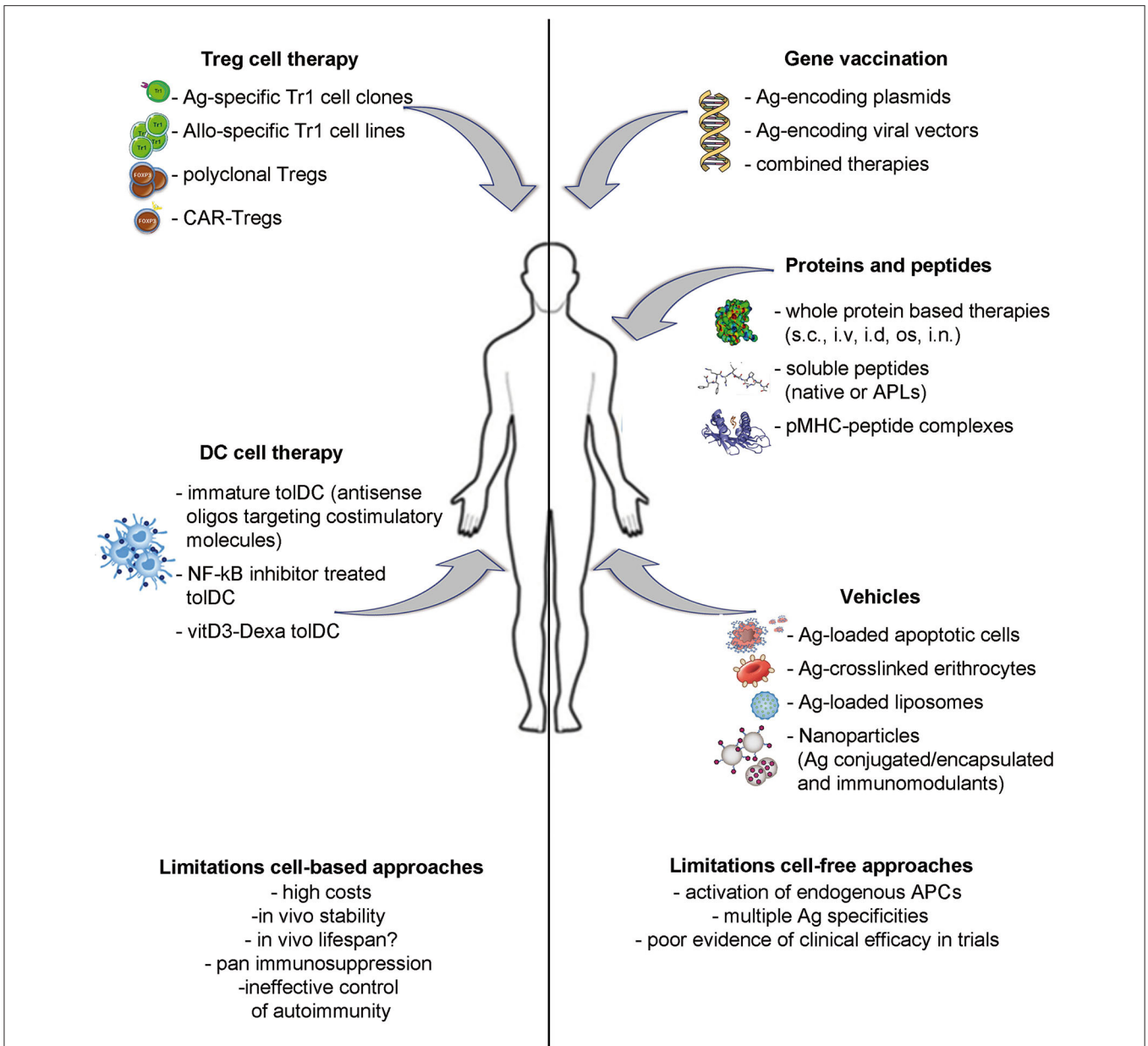


FIGURE 1 | Strategies to induce Ag-specific tolerance in T cell mediated diseases. Approaches under development include: inverse vaccination with autoantigen-encoding DNA or viral vectors; *in vivo* administration of whole Ags, unmodified peptides or altered peptide ligads (APLs); autoantigen-loaded vehicles; transfer of polyclonal or Ag-specific Tregs or of tolerogenic DC loaded with disease-relevant Ags.

TABLE 1 | Clinical trials using antigen-specific approaches.

	Design	Disease	outcome	Trial ID	References
Plasmid DNA	MBP, i.m.	MS Adult	Reduced IFNg-producing $CD4^+$ T cells Decrease of autoantibodies in CNS	NCT00103974	(4)
	MBP, i.m.	MS Adult	No effects	NCT00382629	(5)
	hINS, i.m.	T1D Adult	Increased C-peptide Decreased insulin-reactive $CD8^+$ T cells	NCT00453375	(6)
	hINS, i.m.	T1D Children	Ongoing	NCT03794960	N.A.
	hINS, i.m.	T1D Adult	Ongoing	NCT03794973	N.A.
	PPI + TGF-β1, IL-10, IL-2, s.c.	T1D Adult	Ongoing	NCT04279613	N.A.
Proteins	Ins, oral	T1D Adult	No clinical effects	N.A.	(7)
	Ins, oral	T1D Adult	No clinical effects	IMDIAB trial	(8)
	Ins, oral	T1D Adult	Increased C-peptide in patients > age 20 years	N.A.	(9)
	Ins, oral	FDR	No delay or no T1D prevention	NCT00004984	(10)
	Ins, intranasal	T1D Adult	No T1D prevention Evidence of insulin-specific tolerance	N.A.	(11)
	Ins, intranasal	FDR children	No T1D prevention	NCT00223613	(12)
	Ins, intranasal	FDR	Ongoing	NCT00336674	N.A.
	Ins, oral	FDR	No T1D prevention Modulation of insulin-response	ISRCTN76104595 isrctn.org	(13)
	Ins, oral	FDR	Ongoing	NCT02580877	N.A.
	Ins+ IFA i.m.	T1D Adult	No T1D prevention Induction of insulin-specific Tregs	NCT00057499	(14)
	Ins + MAS-1 i.m.	T1D Adult	ongoing	NCT03624062	N.A.
	GAD-alum s.c.	Newly diagnosed T1D	No clinical effects	NCT00529399	(15)
	GAD-alum s.c.	Newly diagnosed T1D	No clinical effects	NCT00723411	(16)
	GAD-alum s.c.	LADA	No clinical effects	N.A.	(17, 18)
	GAD-alum + Vit D s.c.	LADA	Ongoing	NCT04262479	N.A.
	Myelin, oral	RR-MS	No clinical effects	N.A.	(19)
	Myelin, oral	RR-MS	No clinical effects Induction of myelin-specific TGF-b1+ cells	N.A.	(20)
Peptides	PPI (C19-A3), intradermal	Newly diagnosed T1D	Maintenance of C-peptide over 6-months Increased IL-10-expressing T cells	NCT01536431	(21, 22)
	MBP8298, i.v.	Secondary progressive MS	No stable clinical benefit	NCT00468611	(23, 24)
	Multiple Islet Peptides, intradermal	Newly diagnosed T1D	Not published	NCT02620332	N.A.
	IMCY-0098, s.c.	Newly diagnosed T1D	Not published	NCT03272269	N.A.
	MBP-derived peptide cocktail ATX-MS-1467, intradermal – s.c.	RR-MS	Safety and tolerability No clinical response	NCT01097668	(25)
	MBP-derived peptide cocktail ATX-MS-1467, intradermal	RR-MS	Reduction in MRI lesions	NCT01973491	(26)
	MBP85-99, MOG35-55, and PLP139-155, transdermal	RR-MS	Reduction of clinical outcomes, induction of Tregs	N.A.	(27, 28)
	HLA-DQ2.5-restricted gliadin peptides, intradermal	CD-GFD, HLA-DQ2·5	Unresponsiveness of T cells after gluten challenge	NCT02528799	(29, 30)
	gliadin peptides, intradermal	CD-GFD, HLA-DQ2·5	Ongoing	NCT03644069	N.A.
	APL (NBI-5788), s.c.	MS	Persistent Th2 immune deviation Hypersensitivity	N.A.	(31, 32)
	APL (CGP77116), s.c.	MS	Th1 skewing Disease exacerbation in some patients	NCT00001781	(33)
	APL (NBI-6024), s.c.	Newly diagnosed T1D	No clinical effects	NCT00873561	(34)
	DR2:MBP84-102 (AG284), i.v.	Progressive MS	No clinical effects	N.A.	(35)
	DR2:MOG35,55 (RTL100), i.v.	MS Adult	No clinical effects	N.A.	(36)

(Continued)

TABLE 1 | Continued

	Design	Disease	outcome	Trial ID	References
Peptide coupled with cells	PBMC coupled with a pool of myelin peptides, i.v.	RR-MS Progressive MS	Decrease in antigen-specific T cells	NCT01414634 ETIMS	(37)
	RBC coupled with a pool of myelin peptides, i.v.	RR-MS	Decrease in myelin-specific T cells with an increased Treg frequency	ETIMS^{Red}	(38)
Liposomes Nanoparticles	PLGA-gliadin (TIMP-GLIA) i.v.	CD	Completed results unpublished	NCT03486990	N.A.
	PLGA-gliadin (TIMP-GLIA) i.v.	CD-GFD	Ongoing	NCT03738475	N.A.
Treg-based therapy	Expanded Treg, i.v.	Newly diagnosed T1D children	Short term preservation of C-peptide No long-term effects	ISRCTN06128462 isrctn.org	(39, 40)
	Expanded Treg, i.v.	Newly diagnosed T1D	Short term preservation of C-peptide	NCT01210664	(41)
	Expanded Treg, i.v.	SLE	Terminated due to participant recruitment	NCT02428309	(42)
	Expanded Treg, i.v.	Newly diagnosed T1D	Completed, unpublished	NCT02691247	(42)
	Expanded Treg, i.v.	Autoimmune Hepatitis	Ongoing	NCT02704338	(42)
	Expanded Treg, i.v.	Pemphigus Vulgaris	Ongoing	NCT03239470	(42)
	Expanded Treg, i.v.	IBD	Ongoing	NCT03185000	(42)
	Expanded Treg, i.v.	Alzheimer Disease	Ongoing	NCT03865017	N.A.
	Treg, intravitreous	Bilateral Severe Uveitis	Suspended	NCT02494492	(42)
	Cord-blood Treg, i.v.	Guillain–Barré syndrome	Ongoing	NCT03773328	N.A.
	Expanded Treg + IL-2, i.v.	Newly diagnosed T1D	Ongoing	NCT02772679	N.A.
	Expanded Treg + Liraglutide, i.v.	Newly diagnosed T1D	Ongoing	NCT03011021	N.A.
	Ova-specific Tr1 cell clones, i.v.	Refractory Crohn's	Expansion of OVA-specific Treg Limited clinical responses	CATS1/CATS29	(43)
DC-based therapy	shRNA CD40, CD80 and CD86, i.p.	T1D	Safety and tolerability, no clinical responses	NCT00445913	(44)
	Citrullinated peptide loaded DC, intradermal	RA	Safety and tolerability, no clinical responses	N.A.	(45)
	VitD3/dexa synovial fluid loaded DC, intra-articular	RA	Safety and tolerability, Knee symptoms stabilized in two patients	NCT01352858	(46)
	VitD3 myelin peptides loaded DC, intradermal	MS	Ongoing	NCT02618902	N.A.
	VitD3 myelin peptides loaded DC, intranodal	MS	Ongoing	NCT02903537	(47, 48)
	Peptides loaded TolDC i.v.	MS neuromyelitis optica	Ongoing	NCT02283671	(47–49)
	IFN-α/GM-CS/Dexa DC, intra-articular	RA	Ongoing	NCT03337165	N.A.

MBP, Myelin Basic Protein; PPI, PrePro Insulin; hINS, Insulin; Ins, Insulin; GAD, glutamic acid decarboxylase; APL, Altered Peptide Ligand; PLGA, poly(lactic-co-glycolic acid; VitD3, Vitamin Da; Dexa, dexamethasone; MS, Multiple Sclerosis; T1D, type 1 diabetes; FDR, first-degree-relative; LADA, latent autoimmune diabetes; RR-MS, relapse and remitting MS; CD, Celiac Disease; CD-GFD, Celiac Disease in gluten-free-diet; SLE, Systemic Lupus Erythematosus; IBD, Inflammatory Bowel Disease; RA, Rheumatoid Arthritis; i.m., intramuscular injection; s.c., subcutaneous; i.v., intravenous injection; i.p.,intraperitoneal injection; N.A., not applicable.

cells, and, in case of DNA vectors, translocate to the nucleus for transcription, followed by translation in the cytoplasm and presentation of the Ag in the context of HLA class I molecules. The balance between an inflammatory immune response and the induction of tolerance can be controlled by several factors, including the route of administration, the target tissue, and the vector design. For example, direct transfection or transduction of professional antigen presenting cells (APCs) may result in efficient presentation to Ag-specific $CD8^+$ T cells (50) or, as a consequence of cell death or tissue damage, the Ag may be taken up by professional APCs, processed as exogenous Ags, and presented to Ag-specific $CD4^+$ T cells in the context of HLA class II (51, 52). On the same line, the vector backbone itself may contain immunostimulatory sequences, which could impact on gene expression, intracellular localization of the product and APCs activation *via* TLRs (53). Hence, the activation status of APCs is pivotal for the final outcome of the response: protection vs. tolerance. Two strategies for the delivery of the Ag-coding sequences have been used in preclinical studies, plasmids and viral vectors [reviewed in (42)].

Plasmid DNA

Intramuscular plasmid DNA vaccination has been the most studied, likely due to the short persistence in the host, the low immunogenicity, and the low costs of plasmid production. This strategy was first tested in experimental autoimmune encephalitis (EAE), the murine model of multiple sclerosis (MS): immunization with plasmid encoding for an EAE epitope of

myelin basic protein (MPB) prevented disease development, via T helper (Th)2 cell skewing of the Ag-specific T cell response (54). The initial preclinical studies led to clinical testing of this strategy not only in MS (4, 5), but also in Type 1 Diabetes (T1D) (6) (**Table 1**). A DNA vaccine (BHT-3009, Bayhill Therapeutics) containing full-length sequence of the human MBP was tested in two trials in MS patients (4, 5). In the first trial no severe adverse events were reported. Results indicated a trend of lower lesion activity, reduced IFNγ-producing $CD4^+$ T cells up to 50 weeks after initiation, and a decrease of autoantibodies in the cerebrospinal fluid (4). Nonetheless, in the second trial the intervention did not result in any differences in the time to first relapse, rate of relapses per year, disability progression, and the treatment showed a deleterious effect at high vaccine dose, likely due to a greater percentage of immunostimulatory CpG motifs in the DNA plasmid (5). A similar approach was tested in T1D with a bacterial plasmid encoding for pro-insulin [BHT-3021, Bayhill Therapeutics; (6)]. No serious adverse events were observed, and the treatment resulted in improvement of endogenous insulin production, measured as 28% increase in C-peptide, and decreased frequency of proinsulin-reactive $CD8^+$ T cells (6). Despite encouraging results, insulin requirements did not change substantially, and demonstration of efficacy is still pending. The same product (under the name TOL-3021, Tolerion Inc.) is going to be tested in two distinct phase II trials in T1D children and adults (NCT03794960 and NCT03794973). On the same line, DNA vaccines based on oral administration of recombinant live attenuated bacteria expressing diabetes autoAgs in combination with inhibitory cytokines, such as transforming growth factor (TGF-β1) and IL-10 or with anti-CD3 mAb have also been tested to prevent or revert the onset of diabetes in non obese diabetic (NOD) mice, showing induction of Tregs (both FOXP3-expressing and Tr1 cells) and suppression of autoimmunity (55, 56). A phase I trial will test the safety of subcutaneous injection of a plasmid co-encoding for T1D Ag and adjuvant cytokines (NNC0361-0041: plasmid encoding pre-proinsulin, TGF-β1, IL-10, and IL-2, Novo Nordisk A/S, NCT04279613).

Overall, thus far the plasmid DNA delivery approach showed the ability to skew the immune response, with no evidence of stable tolerance induction. The combination with immunomodulatory cytokines, which should sustain Ag-specific Treg induction, is expected to boost the induction of active tolerance. Results of ongoing clinical trials will shed light on the valuability of this approach.

Viral Vectors

As alternative to plasmids, the use of viral vectors allows to restrict expression of the autoAg to specific tissues and avoid unwanted expression in activated APCs. In this context, the liver is an ideal target, due to its intrinsic tolerogenic properties [reviewed in (57)]. Two types of viral vectors have been used to target gene expression specifically to hepatocytes: the recombinant adeno associated vectors (AAV) and the lentiviral vectors (LVs). Although widely used as vector systems for liver directed *in vivo* gene therapy, few groups explored the use of AAV to induce tolerance to autoAgs in autoimmune diseases. Liver gene therapy with an AAV vector encoding for the full sequence of myelin oligodendrocyte glycoprotein (MOG) prevented development of and reversed preexisting EAE *via* the induction/expansion of Ag-specific $FoxP3^+$ Tregs (58). Earlier studies of intramuscular injection in NOD mice of AAV encoding for glutamic acid decarboxylase (GAD) peptides prevented the development of overt diabetes in NOD mice *via* skewing of Teff cells to Th2 responses, but those studies were not further developed and active tolerance was not demonstrated (59).

The use of LVs to induce Ag-specific tolerance upon liver targeting was also investigated in NOD mice. Intraveneous injection of LV encoding the insulin B chain (InsB) 9–23 epitope led to specific expression of the autoAg in hepatocytes, thanks to the use of tissue-specific promoter and concomitant de-targeting of Ag expression in professional APCs by miR142 target sequences. This treatment prevented diabetes development by induction of Ag-specific $FoxP3^+$ Tregs. Although highly efficient in prevention, the control of overt disease required a combination therapy with anti-CD3 mAb, to block Teff cells from destroying the target organ (60).

Gene vaccination strategies present several advantages in terms of cost-efficient production and long shelf life for plasmid-based vaccines and available (although expensive) large scale and clinical grade protocols for LV production. However, administration of the therapeutic products invariably leads to deleterious activation of professional APCs and the innate immune system (61) and may not be sufficient to counteract the burden of expanded Teff cells with multiple Ag-specificity. The future of these approaches points at combined therapies to overcome these hurdles.

Protein Delivery Approaches

The direct administration of autoAgs in non-inflammatory conditions to induce tolerance in T cell mediated diseases has been widely investigated, especially in EAE and NOD pre-clinical models (62, 63). The underlying idea is that repetitive administration or exposure to large amounts of protein Ag, as whole protein, native or altered peptide alone or combined to carrier complexes, in the absence of pro-inflammatory adjuvants, will favor the deletion or clonal anergy of autoreactive Teff cells and the induction of Ag-specific Tregs, *via* uptake and presentation of the Ag by endogenous tolerogenic APCs (62, 63). In this context the route of administration is a key issue: the positive results obtained in allergic diseases by oral, intranasal and subcutaneous administration of allergens [reviewed in (64)] led to parallel attempts in autoimmune diseases.

Due to the early recognition of insulin epitopes as antigenic targets in NOD mice (65), insulin was the first Ag investigated for the development of protein-based immunotherapy of T1D. Initial promising results in murine models (66–68) led to the clinical testing of oral (7–10) and intranasal insulin [(11, 12), and INITII, NCT00336674], as tolerizing protocols in subjects at risk to develop the disease [(10, 13), NCT00336674 and TN20, NCT02580877] or in recent onset T1D patients (7–9, 11) (**Table 1**). Although results of few trials are still unpublished (NCT00336674; NCT02580877), thus far, none of them resulted in preserved insulin secretion in T1D

patients. Inverse vaccination with InsB has also been tested as intramuscolar injection with Incomplete Freund's Adjuvant [IBS-VS01, (14)]: despite induction of InsB-specific Tregs, C-peptide levels were unaltered by the treatment. A new formulation of the vaccine in combination with MAS-1, an emulsion-based adjuvant, known to promote Th2 responses (69), is currently being tested in a Phase I study (MER3101, NCT03624062). Several trials betted on GAD65 as key Ag and on different routes: Dyamid, a GAD-Alum vaccine, was administered subcutaneous in recent onset T1D (15, 16) and in adults with latent autoimmune diabetes (LADA) (17) without achievement of clinically desirable results (18). Combination of Dyamid with vitamin D in LADA is currently being tested in a Phase II trial (NCT04262479). Similarly, attempts of oral tolerization with myelin Ags in MS, which date back to the early 90's, showed modulation of Ag-specific immune response, but no evidence of efficacy (19, 20).

Peptide Delivery Approaches

In parallel to whole protein-based approaches, administration of peptides derived from disease-causing Ags was also tested both in T1D and MS (**Table 1**). Intradermal administration of a HLA-DR4-restricted native peptide derived from proinsulin (C19-A3) allowed maintenance of C-peptide levels in new-onset T1D over a 6-month treatment and resulted in increased frequencies of IL-10-expressing T cells [MonoPepT1De, (21, 22)]. The HLA-DR2-restricted immunodominant synthetic peptide MBP8298, containing the MBP immune-dominant epitope 85–96, was extensively tested in patients with MS, without stable clinical benefit (23, 24).

Overall these initial peptide-based approaches resulted in modulation of Ag-specific immune responses, but poor clinically relevant results, likely because autoimmune diseases are not caused by single T cell clones, as a result of epitope spreading (70). Given this phenomenon, recent studies have pointed at mixture of peptides from multiple autoAgs for the modulation of autoimmune diseases: in the context of T1D the MultiPepT1De (NCT02620332) and the IMCY-0098 trial (NCT03272269) have been completed, although results are still unpublished. The same approach was tested in MS: following promising results in humanized mice (25), the MBP-derived peptide cocktail ATX-MS-1467 (Aptiope (https://apitope.com/multiple-sclerosis/) was tested for safety and efficacy in relapsing MS patients. Results showed association of treatment with reduction in Magnetic Resonance Imaging (MRI) lesions (26). Moreover, transdermal application of a mixture of 3 myelin peptides showed significant effect in reducing the MRI and clinical outcomes (27) via the induction of Tregs (28). Similarly, NexVax2, composed of three HLA-DQ2.5-restricted immunodominant gliadin peptides, NPL001, NPL002, and NPL003, has been tested in a phase I clinical trial in Celiac Disease (CD) patients. Despite some gluten-related gastrointestinal side effects, the treatment was safe and well tolerated (29, 30). In treated patients functional unresponsiveness of T cells after gluten challenge was observed, indicating induction of tolerance. Currently, a phase II quadruple blind clinical trial (NCT03644069) is underway (71).

Modification of native peptides alters the way peptides interact with TCR and, therefore, influences subsequent T cell activation and T cell fate. Increasing knowledge of both MHC binding registers and TCR interacting residues of peptides allowed the development of altered peptide ligands (APL), with the aim of favoring the expansion and/or induction of Tregs upon peptide recognition [reviewed in (72, 73)]. Following studies in murine disease models, showing that specific APLs were capable of eliciting cytokine release and affecting T cell polarization (74, 75), APLs were tested *in vivo* in autoimmune diseases. Indeed, two altered peptides of MBP83-99 have already been tested in MS. NBI-5788 (Neurocrine Biosciences Inc), in which L-amino acids were changed to D-amino acids at positions 83, 84, 89, 91, was known to stimulate Th2-type responses in MS patients' PBMC (76). Clinical testing confirmed Th2 immune deviation in treated patients (31), but several patients developed hypersensitivity and antibodies that cross-reacted with native MBP83–99 peptide (32). The second MBP-derived APL tested is CGP77116 (Ala D-amino acids at positions 83, 84, 89, 91). It caused a Th1 skewing of $CD4^+$ T cells cross-reacting with the native peptides, thus raising issues on the APL design (33). On the same line, the use of an insulin β-chain-derived APL (NBI-6024) did not improve or maintain beta cell function in recent onset T1D patients (34). Despite the promising results obtained in murine models and the improvements in the development of algorithms for peptide-HLA-binding prediction, thus far clinical trials using APLs were unsuccessful. The design of APLs currently represents a major caveat: the ability to predict the consequence of peptide binding to HLA molecules on APCs or Ag-receptors on T and B cells is still limited and it needs to be empirically determined for each peptide.

Regardless of the type, origin, number of Ags or the route of administration used, the outcome of the administration of whole proteins or peptides is strictly dependent on the activation status of the host's APCs the Ag is binding to. APCs *in vivo* exist in several different flavors (77), expressing different ranges of activatory or inhibitory cell surface molecules and soluble mediators, which play a critical role on the outcome of the cognate T-APC interaction. Peptide-based therapy showed immunological effects, including increased frequency of Treg cells and of IL-10, suggesting modulation of pathogenic responses. The beneficial effects, although observed only short-term after treatment, are compatible with immune tolerance, thus suggesting that endogenous APCs function was modulated, likely indirectly by a bystander suppression mechanism. We believe that to better sustain long term tolerance protein or peptide based approaches could benefit from strategies designed to keep APCs in check.

One of the strategies tested to address this limitation is the injection of soluble peptide-MHC (pMHC) complexes to target directly T cells. Vaccination with pMHC complexes is predicted to induce tolerance either by deletion of naive and memory Teff cells that recognize the self-peptide, or by induction of Tregs (78). This strategy was applied in preclinical models of Myastenia Gravis (79), in EAE (80–82) and in NOD mice (83) resulting in reduction of T cell responsiveness. Phase I trials were performed

in MS patients (35, 36), but further testing is necessary to assess clinical efficacy.

The experience with administration of soluble Ags was further developed using different types of vehicles designed to deliver the Ag specifically to steady-state or tolerogenic APCs, as outlined below.

VEHICLE APPROACHES TO DELIVER ANTIGENS

A number of different approaches to deliver Ag specifically to APCs *in vivo* have been investigated in pre-clinical studies and some of them have been translated into clinical application.

Peptide Coupled to Cells

The first approach tested was the intravenous administration of antigenic peptides cross-linked to peripheral blood or splenic leukocytes using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI), which promotes Ag coupling and induces cell apoptosis (Ag-SP) (84). Once injected *in vivo,* apoptotic Ag-SP are taken up by APCs and trigger the production and secretion of IL-10 and TGF-β and the up-regulation of PD-L1, leading to T cell anergy and apoptosis of pathogenic T cells and Treg induction (84). The efficacy of Ag-SP has been demonstrated in pre-clinical models, including EAE and NOD mice [reviewed in (84)]. The translation to the clinic of this approach was the administration of autologous peripheral blood cells coupled with seven MS-related peptides to MS patients in a Phase I-II clinical trial (ETIMS) (**Table 1**). Results demonstrated the feasibility, safety and tolerability of the treatment, and a decrease in Ag-specific T cell responses (37).

An alternative approach to deliver Ags to APCs in a tolerogenic manner is the administration of Ag-loaded erythrocytes, thus exploiting the natural tolerization mechanisms of dying red blood cells (85). To facilitate the binding of peptide Ags to erythrocytes, peptides were designed to contain the 12aa sequence ERY1 that binds to glycophorin A or sortase A on erythrocytes. Testing in EAE, in NOD and in transgenic mice demonstrated the deletion of Ag-specific T cells *in vivo* (86–88). Using this approach a phase Ib trial (ETIMS^{Red}) has been completed; mechanistic studies demonstrated a reduction in myelin-specific T cell responses with an increased frequency of Tr1 and nTreg cells, thus pointing toward active induction of immune tolerance (38). Possible clinical translation of the erythrocyte binding technology is currently pursued by Anokion (www.anokion.com).

Liposomes and Nanoparticle

As an alternative, to mimic the features of apoptotic cells, liposomes containing phosphatidylserine have been developed and loaded with antigenic peptides. Injection of liposomes loaded with MS-related peptides reduced symptoms in the EAE model (89). Instead, phosphatidycholine liposomes loaded with Ag and NF-kB inhibitors reduced disease severity in a mouse model of arthritis (90). Poly-(lactic-co-glycolic acid) (PLGA) microspheres carrying anti-sense oligonucleotides for the costimulatory molecules CD40, CD80, and CD86 delivered to NOD mice prevented T1D development (91, 92). Notably, the Authors showed that the Ag was not required to elicit Ag-specific Tregs, since, upon microsphere administration, DC migrate from the site of injection to the pancreatic lymph nodes, where auto-Ags are captured and presented to T cells, thus leading to Ag-specific Treg induction (91). This approach is under development for the treatment of T1D (DiaVac. Inc, https://www.angelmd.co/en/startups/diavacsinc).

The discovery that polymeric biodegradable nanoparticles (NPs) could efficiently deliver molecules *in vivo*, prompted investigators to develop NPs suitable for tolerance induction. PLGA-NPs can encapsulate immune-modulatory agents, such as rapamycin, alone or in combination with peptide Ags. Once injected *in vivo* these NPs target DC, thus allowing Ag-presentation in a tolerogenic manner (93). Pre-clinical studies showed that *in vivo* delivery of PLGA-NPs containing MS-related peptide Ags prevents and treats EAE by up-regulating PD-L1 on APCs and inhibiting the production of pro-inflammatory cytokines by Ag-specific pathogenic T cells (93). PLGA particles encapsulating gliadin (TIMP-GLIA) were developed for application as a therapy for CD and tested in a Phase I clinical trial (NCT03486990). The results of this trial are yet to be published, and the Phase II trial is currently underway (NCT03738475).

The tolerogenic effects of NPs depend on size, which dictates their trafficking and biodistribution: (i) particles smaller than 6 nm drain to the blood; (ii) particles larger than 9 nm preferentially drain to lymphatics; (iii) particles in the range of 20–100 nm accumulate in liver sinusoidal endothelial cells (LSECs) or macrophages; (iv) particles from 100 to 200 nm can traffic to the spleen and liver; (v) particles from 200 nm to 5 μm accumulate in the spleen. Moreover, NPs biodistribution is also affected by the route of administration: intravenous injection targets APCs in the spleen and liver, whereas upon subcutaneous injection NPs are taken up by DC that accumulate in draining lymph nodes (94). The ability of LSECs to promote induction of FoxP3^{+} Tregs, prompted the development of NPs to deliver Ags to LSECs for autoimmune disease treatment (95). NP-based autoAg delivery to LSECs prevented the onset of clinical EAE and, in therapeutic settings, mice with already established EAE improved rapidly (95).

More recently, a further evolution on the NP approach to deliver Ag and promote tolerance was described by Santamaria et al. (96, 97). This approach consists on coating NPs with MHC class I or MHC class II molecules coupled with antigenic peptides (pMHC-coated NPs) (98). In pre-clinical models, the administration of pMHC-coated NPs promoted the differentiation of Ag-specific Tr1 cells and the conversion of Ag-specific Th1 cells into Tr1 cells, following massive expansion. Expanded Tr1 cells were activated by autologous APCs presenting the cognate Ag and induced bystander IL-10-mediated suppression (98). These pMHC-coated NPs (Navacims^{TM}) have been validated in different pre-clinical models of autoimmunity (96, 97) and are currently under clinical development.

The application of nanotechnology to advance treatment of autoimmunity is likely to undergo major development in coming

years. Nanotechnology will create new materials for NP-related products. However, NPs are highly reactive, leading to their potentially harmful interaction with biological systems and the environment, thereby increasing the risk of toxicity. Detection of adverse effects is complex, since they depend on the route of administration, doses and size of NPs. NPs accumulate in the reticuloendothelial system and their long-term effects are not yet fully elucidated. Moreover, the small size of nanomaterial allows their penetrance into deeper areas of biological systems that are usually inaccessible to larger particles. Thus, due to the different properties of NPs, their application for therapeutic purposes, especially the long-term effect on the immune system, requires further attention and research (99, 100).

CELL-BASED APPROACHES

Cell-based therapies are clinically attractive for promoting or restoring tolerance in T cell mediated diseases as they can theroretically control several inflammatory cells, including T and B lymphocytes, NK cells and APCs, leading to the control of unwanted immune responses. Therapies based on adoptive transfer of regulatory cells (T, macrophages, and DC) entered the clinical trial arena in the last years with the goal to investigate the safety and feasibility of the approach, and several studies are still ongoing.

Treg-Based Therapies

The increasing knowledge on the biology of Tregs, on their mode of action and their ability to control autoimmune responses when adoptively transferred *in vivo* in pre-clinical models of autoimmunity allowed the growth of a number of clinical trials to investigate the safety and feasibility of the approach (42, 101). The literature on Treg cell therapy is extensive and will not be reviewed here in depth. Tregs were first used in clinical trials to treat patients with graft vs. host disease (GvHD) after hematopoietic stem cell transplantation (HSCT). Results demonstrated that Tregs are safe, with some concern about the occurrence of mild to moderate infections (101). Treg therapy is currently applied to reduce dependency on immunosuppressive drugs in patients after organ transplantation (101, 102). In the context of autoimmune diseases both $FOXP3^+$ Tregs and Tr1 cells have been tested in clinical trials (**Table 1**). The infusion of *ex-vivo* expanded polyclonal $FOXP3^+$ Tregs in patients with recently diagnosed T1D showed improved beta-cell function and reduced exogenous insulin requirement only short-term (39–41). The limited efficacy of Treg-based immunotherapy in T1D may depend on the limited number of residual functional beta-cells at time of treatment, the inadequate availability of IL-2 *in vivo* (40), or, more importantly, on the lack of antigen-specificity of the infused Tregs. A number of clinical trials with expanded autologous Tregs are ongoing, have been closed, or have been completed but results have not been published yet [NCT02428309; NCT02494492; NCT02691247; NCT02704338; NCT03239470; NCT03185000; NCT03773328; NCT03865017; (42)]. Pre-clinical studies indeed showed that Ag-specificity may offer an advantage for Treg function compared to polyclonal Tregs (103). The first experience with Ag-specific Tregs was in Crohn's disease: ovalbumin-specific Tr1 cells (Ovasave®) expanded *in vitro* were infused in patients, who ingested ovalbumin to allow Treg activation and inhibitory function in the gut, with no side effects, but limited clinical effects (43). Beside the use of T cell clones, several other approaches have been investigated and applied to generate Ag-specific Tregs. The most advanced strategies were applied to the transplantation area: alloAg-specific Tregs can be generated using tolerogenic DC (104–106) or by engineering Tregs with a chimeric antigen receptor (CAR) recognizing HLA-A2 (107, 108). These approaches are currently under clinical investigation (NCT03198234; TX200, www.sangamo.com). Translation of the latter strategy to autoimmune settings is more challenging because (i) the Ags inducing the disease are often unknown; (ii) Tregs and pathogenic T cells are driven by different epitopes; and (iii) while disease progresses, epitope spreading occurs.

Results of the pioneer trials of adoptive Treg cell therapies in transplantation and T1D taught the field that transfer of Tregs alone may not be sufficient to control immune responses in the long-term, thus combined therapies with growth factors or repetitive Treg injections are currently under investigation. Based on the evidence that low doses of IL-2 can increase the endogenous pool of Tregs (109), the combination of a single infusion of autologous *ex-vivo* expanded polyclonal Tregs with IL-2 or with Liraglutide in patients with T1D is currently under clinical testing (NCT02772679 and NCT03011021).

Overall, Treg-based clinical trials demonstrated the safety and feasibility of the approach with some clinical benefit. However, several open issues remain to be solved specifically in the application of polyclonal *ex-vivo* expanded Tregs: (i) their potential to mediate pan immunosuppression *in vivo*, due to the phenomenon of bystander immune suppression; (ii) their intrinsic instability when exposed to strong inflammatory conditions *in vivo,* thereby the risk of pathogenic conversion and exacerbation of the disease; (iii) the overall impact of long-lasting Tregs on infections and malignancies (110).

DC-Based Approaches

It is now widely accepted that DC, either naturally arising or experimentally induced, play a critical role in the maintenance of tissue homeostasis and in promoting tolerance [reviewed in (111–113)], thus acting as regulatory cells. DC can acquire regulatory capacity upon treatment with immunosuppressive mediators, genetic manipulation or signals from other immune cells (114). DC with regulatory properties are generally indicated as tolerogenic DC (tolDC): they present Ags and prime Ag-specific T cells, while down-regulating the expression of costimulatory molecules and pro-inflammatory cytokines, and up-regulating the expression of inhibitory and/or modulatory receptors and anti-inflammatory cytokines. As a result, priming or activation of T cells by tolDC leads to induction of Ag-specific Tregs (114). On the other hand, DC sense environmental signals, which can impact their maturation and activation status and can modulate their microenvironment by release of soluble factors, thus indirectly impacting the outcome of Ag recognition by T cells.

A better understanding of the biology of tolDC and the development of protocols for the generation of tolDC *in vitro*, opened the possibility to translate their use as immunotherapy

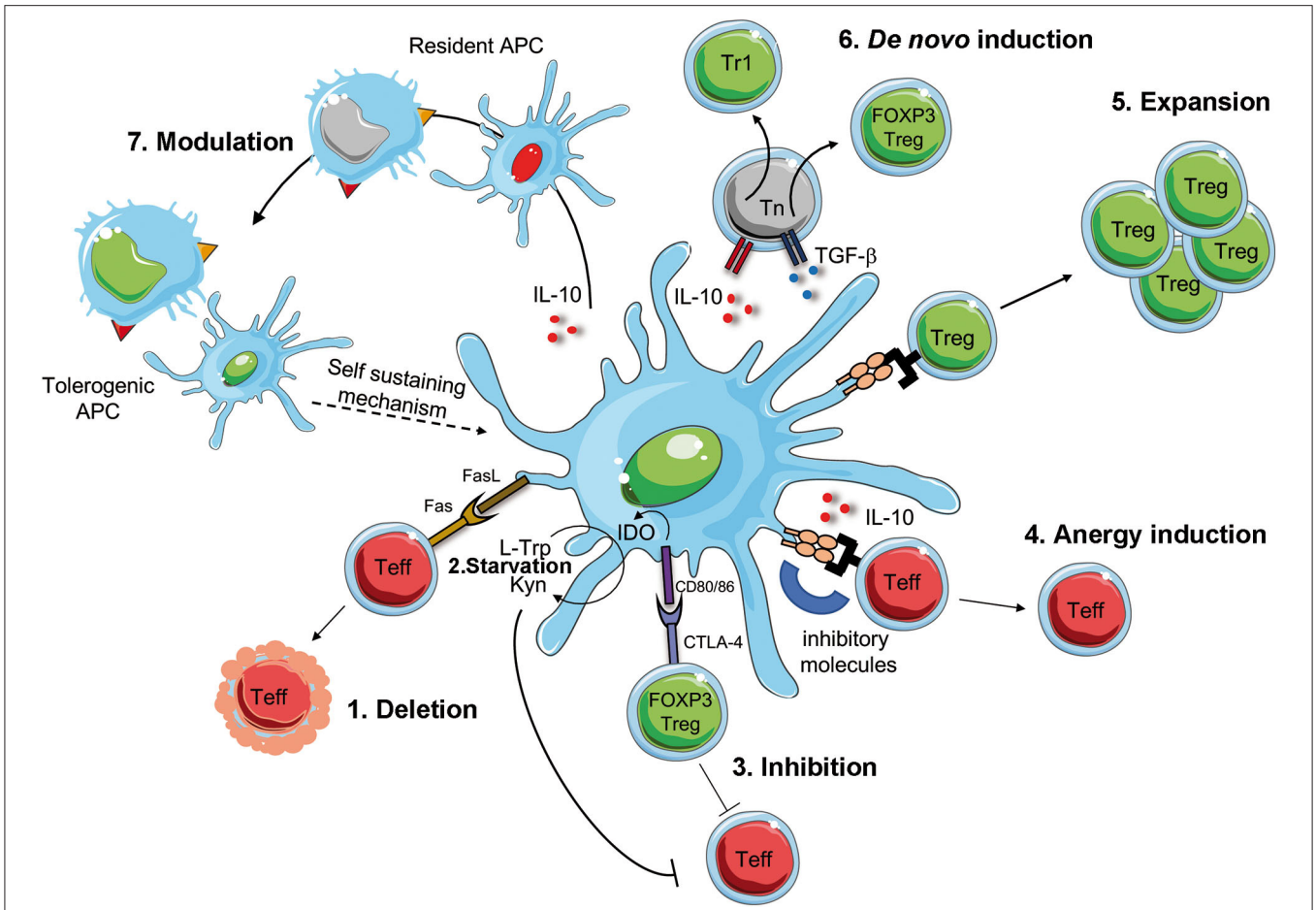


FIGURE 2 | Mechanism of DC-mediated tolerance. Tolerogenic (Tol)DC promote deletion or modulate Teff cells via Fas/FasL interaction [1], starvation of Teff cells *via* IDO production that degrades tryptophan (L-Trp) into kynurenine (Kyn) [2]. IDO is induced by the interaction between CD80/CD86 on tolDC and CTLA-4 on regulatory T cell (FOXP3 Treg), which concur to the suppression of Teff cells [3]. The interaction of inhibitory molecules on tolDC and Teff cells in the presence of IL-10 secretion promotes T cell anergy [4]. TolDC favor the activation and expansion of pre-existing Tregs [5] of *de novo* induction of FOXP3 Treg of Tr1 cells [6]. Finally, surface expression of inhibitory molecules and secretion of regulatory mediators promote the conversion of resident APCs into tolerogenic APCs, which sustain tolerance [7]. Teff, effector T cells; CTLA-4, cytotoxic T-lymphocyte antigen-4; IDO, indoleamine 2,3-dioxygenase; L-Trp, L-tryptophan, Kyn, kynurenins; Fas, first apoptosis signal; FasL, Fas ligand.

in clinical trials for immune-mediated diseases (115, 116). These therapies are not simple alternatives to Treg-based therapies, but they are complementary. *Ex-vivo* generated tolDC have the potential to induce, enhance, or restore Ag-specific tolerance *in vivo* since, once loaded with Ags, they act in an Ag-specific manner. TolDC can regulate pathogenic T cell responses *via* several mechanisms, including T cell deletion or inhibition, induction of T cell anergy, *de novo* Treg generation or expansion of pre-existing Tregs, and modulation of APCs (**Figure 2**). TolDC can delete Teff cells by inducing T cell apoptosis *via* Fas/FasL pathway. Furthermore, tolDC can inhibit Teff cell function either directly, *via* production of the enzyme indoleamine 2,3-dioxygenase (IDO), which degrades the amino acid tryptophan (L-Trp) causing starvation of pathogenic T cells (117), or indirectly, by activating pre-existing Tregs *via* interaction between CD80/CD86 and CTLA-4 to exert their suppressive function. TolDC can also promote the induction of T cell anergy into Teff cells *via* the secretion of anti-inflammatory cytokines, such as IL-10, or signals *via* inhibitory molecules, such as HLA-G and ILT3/4 (104, 118). Moreover, tolDC promote the expansion of pre-existing Tregs and *de novo* induction of both Tr1 cells and FOXP3^{+} Tregs, *via* the secretion of IL-10, TGF-β and active kynurenines, products of IDO-mediated L-Trp degradation (119). Finally, tolDC, via the expression and secretion of regulatory molecules, can also modulate APCs, rendering them pro-tolerogenic (e.g., modulation of resident macrophages into an M2 phenotype, or dampening the maturation of resident DC), a process that generates a self-sustaining tolerogenic microenvironment, which can promote long-term tolerance. Beside exerting their effect on immune cells, tolDC secrete several factors (e.g. pro-angiogenic cytokines) which promote tissue repairing and regeneration (**Figure 2**). Altogether, these properties rendered tolDC the cells of choice to restore tolerance in autoimmune diseases.

Pioneer clinical trials with adoptive transfer of tolDC demonstrated the safety, feasibility and efficacy of the

treatment and some clinical benefits [reviewed in (115)] (**Table 1**). Several tolerogenic approaches have been used in the past. In the first-in-man study, autologous tolDC treated with antisense oligonucleotides targeting CD40, CD80 and CD86 to maintain their immature state were infused in T1D patients [NCT00445913, (44)]. The group of Thomas treated DC with a nuclear factor-kB (NF-kB) inhibitor and pulsed them with citrullinated peptide Ags before injection into RA patients (45). More recently DC differentiated with vitamin D3 and dexamethasone alone or in combination have been or are currently used to treat RA, Crohn's disease, and MS patients [NCT01352858-AutoDECRA (46); NCT02618902-MS-tolDC; NCT02283671-TolDecEM/NMO (47–49); NCT02903537-Tolervit-MS (48, 49); NCT03337165-TolDCfoRA; (120)].

Despite these encouraging results, phase II/III clinical trials are needed to address several open issues and to allow comparison to current available treatments. Indeed, a number of open questions remain before tolDC-based therapies can be routinely used to treat or cure autoimmune diseases (101, 116). A variety of routes for tolDC administration have been tested in the past, including intradermal, intraperitoneal, intravenous and intra-articular (121). These administration routes are indeed required to allow tolDC to reach the relevant draining lymph nodes or the disease-specific site of inflammation. However, if direct administration to the relevant tissue is challenging, such as in the case of T1D, intraperitoneal administration has been preferred.

As for any Ag-specific approach for tolerance induction, an additional major hurdle in developing an effective tolDC-based therapy is the selection of the Ag critical for a given disease. As in the case of peptide-based approaches, the use of broad spectrum disease-related peptides has been postulated to overcome this limitation [reviewed in (101)]. Interestingly, in the context of T1D the identification of neoepitopes opened new perspectives in the field. The peptides characterized by improved MHC binding register, such as the insulin peptide $InsB_{9-23}$ with combined substitutions in positions 14, 21, and 22 (122), those generated by fusion of peptides, such as the Hybrid Insulin Peptides (HIPs) (123), or by aberrant translation, such as INS-DriP peptide (124), have been shown to trigger strong specific T cell responses. These highly immunogenic peptides presented by tolDC are promising tools for the reprogramming of pathogenic T cells and induction of tolerance in T1D.

Besides the critical issues discussed above some additional considerations should be taken into account when designing tolDC-based therapies: (i) the necessity of multiple cell infusions to allow the induction of the self-sustained mechanisms described above will invariably lead to high manufacturing costs; (ii) the generation of autologous tolDC implies the use of patient-derived monocytes, which may not be as functional as those isolated from healthy subjects (105); (iii) the stability of the cell product to be infused must be evaluated for limiting *in vivo* side effects or disease exacerbation.

CONCLUSIVE REMARKS

In recent years the development of *in vivo* and *ex-vivo* Ag-specific approaches to modulate detrimental immune responses has made striking progress. Results obtained in Phase I/II demonstrated the safety and tolerability of the approaches with, thus far, limited clinical responses. Phase II/III clinical trials will help in defining whether the strategies outlined here will reach the goal of completely reversing the course of T cell mediated diseases.

Overall, results obtained thus far highlighted common requirements for achieving the desired effectiveness of the Ag-specific based therapy, either peptide or protein delivery, or the vehicle strategies to delivery Ags or the regulatory cell-based approaches: the repetitive administrations and the use of multiple Ags to effectively activate the tolerogenic branch of the immune response and to tackle the epitope spreading, respectively. Moreover, the selection of the most suitable epitope/s to be used might be challenging, because different patients may display preferential response to specific Ags. This issue opens the need for the identification of peptide Ags that can be used across different HLA-type patients [e.g., (21, 22)] or for deeper characterization of patients' reactivity before enrollment in trials.

The field is rapidly evolving, and the upcoming clinical trials will confirm the safety and feasibility and will shed light on the efficacy of Ag-specific approaches. Several issues remain to be clarified for each of the approaches in the pipeline. Regardless of the tolerogenic approach used, one of the open questions in the field of tolerance induction is the definition of common parameters to monitor the response to treatment, and to allow comparison of different approaches. In the context of cell-based tolerance-inducing therapies an initiative of the European scientific community brought together the leader scientists in the field of cell-based therapies and autoimmune diseases under the umbrella of the European Cooperation in Science and Technology (COST). The main objective of A-FACTT Action was to coordinate efforts to minimize overlap and maximize comparison of the diverse cell-based approaches through establishment of consensus monitoring parameters (https://www.cost.eu/actions/BM1305/#tabs|Name:overview). More of such initiatives could help the field to address this relevant point. On the same line, the definition of "tolerogenic treatment" should be unambiguouosly referred to therapies inducing long-term active tolerance. Indeed, several treatments have been shown to modulate immune responses in the short term, but fail in controlling disease signs long-term. Tolerogenic therapies should promote long-lasting effects, and this can be achieved by different mode of action, including the conversion of pathogenic Teff cells into Tregs, or the *de novo* induction of Tregs. As discussed above, we believe that modulated DC, or APCs, represent the population of cells able to prevent activation of pathogenic Teff cells, to promote *de novo* induction of Tregs, and to re-educate Teff cells to become Tregs, thus maintaining tolerance long-term. To achieve these long lasting effects possible repetitive injection of the tolerogenic treatment might be required. Based on the central role of APCs in determining the outcome of Ag-specific

T cell activation, inverse vaccination strategies are unlikely to be successful, unless the underlying mechanism allows boosting of the immunomodulatory properties of DC or, more generally, of APCs.

AUTHOR CONTRIBUTIONS

LP and SG wrote the manuscritpt. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Telethon Foundation (Tele20-G1 to SG) and the Italian Ministry of Health (RF-2016- 02361372 to SG).

ACKNOWLEDGMENTS

We thank Giada Amodio for critical reading of the manuscript.

REFERENCES

1. Steinman L. Inverse vaccination, the opposite of Jenner's concept, for therapy of autoimmunity. *J Intern Med.* (2010) 267:441–51. doi: 10.1111/j.1365-2796.2010.02224.x
2. Romano M, Fanelli G, Albany CJ, Giganti G, Lombardi G. Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. *Front Immunol.* (2019) 10:43. doi: 10.3389/fimmu.2019.00043
3. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M, Gagliani N. The Biology of T Regulatory Type 1 Cells and Their Therapeutic Application in Immune-Mediated Diseases. *Immunity*. (2018) 49:1004–19. doi: 10.1016/j.immuni.2018.12.001
4. Bar-Or A, Vollmer T, Antel J, Arnold DL, Bodner CA, Campagnolo D, et al. Induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled phase 1/2 trial. *Arch Neurol.* (2007) 64:1407–15. doi: 10.1001/archneur.64.10.nct70002
5. Garren H, Robinson WH, Krasulova E, Havrdova E, Nadj C, Selmaj K, et al. Phase 2 trial of a DNA vaccine encoding myelin basic protein for multiple sclerosis. *Ann Neurol.* (2008) 63:611–20. doi: 10.1002/ana.21370
6. Roep BO, Solvason N, Gottlieb PA, Abreu JRF, Harrison LC, Eisenbarth GS, et al. Plasmid-encoded proinsulin preserves C-peptide while specifically reducing proinsulin-specific CD8(+) T cells in type 1 diabetes. *Sci Transl Med.* (2013) 5:191ra82. doi: 10.1126/scitranslmed.3006103
7. Chaillous L, Lefevre H, Thivolet C, Boitard C, Lahlou N, Atlan-Gepner C, et al. Oral insulin administration and residual beta-cell function in recent-onset type 1 diabetes: a multicentre randomised controlled trial. Diabete Insuline Orale group. *Lancet.* (2000) 356:545–9. doi: 10.1016/S0140-6736(00)02579-4
8. Pozzilli P, Pitocco D, Visalli N, Cavallo MG, Buzzetti R, Crino A, et al. No effect of oral insulin on residual beta-cell function in recent-onset type I diabetes (the IMDIAB VII). IMDIAB Group. *Diabetologia.* (2000) 43:1000–4. doi: 10.1007/s001250051482
9. Ergun-Longmire B, Marker J, Zeidler A, Rapaport R, Raskin P, Bode B, et al. Oral insulin therapy to prevent progression of immune-mediated (type 1) diabetes. *Ann N Y Acad Sci.* (2004) 1029:260–77. doi: 10.1196/annals.1309.057
10. Skyler JS, Krischer JP, Wolfsdorf J, Cowie C, Palmer JP, Greenbaum C, et al. Effects of oral insulin in relatives of patients with type 1 diabetes: The Diabetes Prevention Trial–Type 1. *Diabetes Care.* (2005) 28:1068–76. doi: 10.2337/diacare.28.5.1068
11. Fourlanos S, Perry C, Gellert SA, Martinuzzi E, Mallone R, Butler J, et al. Evidence that nasal insulin induces immune tolerance to insulin in adults with autoimmune diabetes. *Diabetes.* (2011) 60:1237–45. doi: 10.2337/db10-1360
12. Nanto-Salonen K, Kupila A, Simell S, Siljander H, Salonsaari T, Hekkala A, et al. Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial. *Lancet.* (2008) 372:1746–55. doi: 10.1016/S0140-6736(08)61309-4
13. Bonifacio E, Ziegler AG, Klingensmith G, Schober E, Bingley PJ, Rottenkolber M, et al. Effects of high-dose oral insulin on immune responses in children at high risk for type 1 diabetes: the Pre-POINT randomized clinical trial. *JAMA.* (2015) 313:1541–9. doi: 10.1001/jama.2015.2928
14. Orban T, Farkas K, Jalahej H, Kis J, Treszl A, Falk B, et al. Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy. *J Autoimmun.* (2010) 34:408–15. doi: 10.1016/j.jaut.2009.10.005
15. Wherrett DK, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, et al. Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. *Lancet.* (2011) 378:319–27. doi: 10.1016/S0140-6736(11)60895-7
16. Ludvigsson J, Krisky D, Casas R, Battelino T, Castano L, Greening J, et al. GAD65 antigen therapy in recently diagnosed type 1 diabetes mellitus. *N Engl J Med.* (2012) 366:433–42. doi: 10.1056/NEJMoa1107096
17. Krause S, Landherr U, Agardh CD, Hausmann S, Link K, Hansen JM, et al. GAD autoantibody affinity in adult patients with latent autoimmune diabetes, the study participants of a GAD65 vaccination trial. *Diabetes Care.* (2014) 37:1675–80. doi: 10.2337/dc13-1719
18. Beam CA, MacCallum C, Herold KC, Wherrett DK, Palmer J, Ludvigsson J, et al. GAD vaccine reduces insulin loss in recently diagnosed type 1 diabetes: findings from a Bayesian meta-analysis. *Diabetologia.* (2017) 60:43–9. doi: 10.1007/s00125-016-4122-1
19. Weiner HL, Mackin GA, Matsui M, Orav EJ, Khoury SJ, Dawson DM, et al. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science.* (1993) 259:1321–4. doi: 10.1126/science.7680493
20. Hafler DA, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Fukaura H. Oral administration of myelin induces antigen-specific TGF-beta 1 secreting T cells in patients with multiple sclerosis. *Ann N Y Acad Sci.* (1997) 835:120–31. doi: 10.1111/j.1749-6632.1997.tb48623.x
21. Thrower SL, James L, Hall W, Green KM, Arif S, Allen JS, et al. Proinsulin peptide immunotherapy in type 1 diabetes: report of a first-in-man Phase I safety study. *Clin Exp Immunol.* (2009) 155:156–65. doi: 10.1111/j.1365-2249.2008.03814.x
22. Alhadj Ali M, Liu YF, Arif S, Tatovic D, Shariff H, Gibson VB, et al. Metabolic and immune effects of immunotherapy with proinsulin peptide in human new-onset type 1 diabetes. *Sci Transl Med.* (2017) 9:eaaf7779. doi: 10.1126/scitranslmed.aaf7779
23. Warren KG, Catz I, Ferenczi LZ, Krantz MJ. Intravenous synthetic peptide MBP8298 delayed disease progression in an HLA Class II-defined cohort of patients with progressive multiple sclerosis: results of a 24-month double-blind placebo-controlled clinical trial and 5 years of follow-up treatment. *Eur J Neurol.* (2006) 13:887–95. doi: 10.1111/j.1468-1331.2006.01533.x
24. Freedman MS, Bar-Or A, Oger J, Traboulsee A, Patry D, Young C, et al. A phase III study evaluating the efficacy and safety of MBP8298 in secondary progressive MS. *Neurology.* (2011) 77:1551–60. doi: 10.1212/WNL.0b013e318233b240
25. Streeter HB, Rigden R, Martin KF, Scolding NJ, Wraith DC. Preclinical development and first-in-human study of ATX-MS-1467 for immunotherapy of MS. *Neurol Neuroimmunol Neuroinflamm.* (2015) 2:e93. doi: 10.1212/NXI.0000000000000093
26. Chataway J, Martin K, Barrell K, Sharrack B, Stolt P, Wraith DC, et al. Effects of ATX-MS-1467 immunotherapy over 16 weeks in relapsing multiple sclerosis. *Neurology.* (2018) 90:e955–62. doi: 10.1212/WNL.0000000000005118
27. Walczak A, Siger M, Ciach A, Szczepanik M, Selmaj K. Transdermal application of myelin peptides in multiple sclerosis treatment. *JAMA Neurol.* (2013) 70:1105–9. doi: 10.1001/jamaneurol.2013.3022

28. Jurynczyk M, Walczak A, Jurewicz A, Jesionek-Kupnicka D, Szczepanik M, Selmaj K. Immune regulation of multiple sclerosis by transdermally applied myelin peptides. *Ann Neurol.* (2010) 68:593–601. doi: 10.1002/ana.22219
29. Tye-Din JA, Stewart JA, Dromey JA, Beissbarth T, van Heel DA, Tatham A, et al. Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. *Sci Transl Med.* (2010) 2:41ra51. doi: 10.1126/scitranslmed.3001012
30. Daveson AJM, Ee HC, Andrews JM, King T, Goldstein KE, Dzuris JL, et al. Epitope-specific immunotherapy targeting CD4-positive T cells in celiac disease: safety, pharmacokinetics, and effects on intestinal histology and plasma cytokines with escalating dose regimens of Nexvax2 in a randomized, double-blind, placebo-controlled phase 1 study. *EBioMedicine.* (2017) 26:78–90. doi: 10.1016/j.ebiom.2017.11.018
31. Kim HJ, Antel JP, Duquette P, Alleva DG, Conlon PJ, Bar-Or A. Persistence of immune responses to altered and native myelin antigens in patients with multiple sclerosis treated with altered peptide ligand. *Clin Immunol.* (2002) 104:105–14. doi: 10.1006/clim.2002.5258
32. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med.* (2000) 6:1176–82. doi: 10.1038/80525
33. Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med.* (2000) 6:1167–75. doi: 10.1038/80516
34. Walter M, Philotheou A, Bonnici F, Ziegler AG, Jimenez R, Group NBIS. No effect of the altered peptide ligand NBI-6024 on beta-cell residual function and insulin needs in new-onset type 1 diabetes. *Diabetes Care.* (2009) 32:2036–40. doi: 10.2337/dc09-0449
35. Goodkin DE, Shulman M, Winkelhake J, Waubant E, Andersson P, Stewart T, et al. A phase I trial of solubilized DR2:MBP84-102 (AG284) in multiple sclerosis. *Neurology.* (2000) 54:1414–20. doi: 10.1212/WNL.54.7.1414
36. Yadav V, Bourdette DN, Bowen JD, Lynch SG, Mattson D, Preiningerova J, et al. Recombinant T-cell receptor ligand (RTL) for treatment of multiple sclerosis: a double-blind, placebo-controlled, phase 1, dose-escalation study. *Autoimmune Dis.* (2012) 2012:954739. doi: 10.1155/2012/954739
37. Lutterotti A, Yousef S, Sputtek A, Sturner KH, Stellmann JP, Breiden P, et al. Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. *Sci Transl Med.* (2013) 5:188ra75. doi: 10.1126/scitranslmed.3006168
38. Lutterotti A., Ludersdorfer T.H., Docampo M.J., Hohmann M., Selles Moreno C., Hayward-Koennecke H, et al. *Establish Tolerance in MS With Myelin-Peptide Coupled Red Blood Cells - The Phase Ib ETIMSredtrial*. Stockholm: ECTRIMS Online Library (2019). p. 279584.
39. Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Techmanska I, Juscinska J, et al. Administration of CD4+CD25highCD127-regulatory T cells preserves beta-cell function in type 1 diabetes in children. *Diabetes Care.* (2012) 35:1817–20. doi: 10.2337/dc12-0038
40. Marek-Trzonkowska N, Mysliwiec M, Iwaszkiewicz-Grzes D, Gliwinski M, Derkowska I, Zalinska M, et al. Factors affecting long-term efficacy of T regulatory cell-based therapy in type 1 diabetes. *J Transl Med.* (2016) 14:332. doi: 10.1186/s12967-016-1090-7
41. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med.* (2015) 7:315ra189. doi: 10.1126/scitranslmed.aad4134
42. Ferreira LMR, Muller YD, Bluestone JA, Tang Q. Next-generation regulatory T cell therapy. *Nat Rev Drug Discov.* (2019) 18:749–69. doi: 10.1038/s41573-019-0041-4
43. Desreumaux P, Foussat A, Allez M, Beaugerie L, Hebuterne X, Bouhnik Y, et al. Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology.* (2012) 143:1207–17 e1–2. doi: 10.1053/j.gastro.2012.07.116
44. Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care.* (2011) 34:2026–32. doi: 10.2337/dc11-0472
45. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci Transl Med.* (2015) 7:290ra87. doi: 10.1126/scitranslmed.aaa9301
46. Bell GM, Anderson AE, Diboll J, Reece R, Eltherington O, Harry RA, et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann Rheum Dis.* (2017) 76:227–34. doi: 10.1136/annrheumdis-2015-208456
47. Zubizarreta I, Florez-Grau G, Vila G, Cabezon R, Espana C, Andorra M, et al. Immune tolerance in multiple sclerosis and neuromyelitis optica with peptide-loaded tolerogenic dendritic cells in a phase 1b trial. *Proc Natl Acad Sci USA.* (2019) 116:8463–70. doi: 10.1073/pnas.1820039116
48. Willekens B, Presas-Rodriguez S, Mansilla MJ, Derdelinckx J, Lee WP, Nijs G, et al. Tolerogenic dendritic cell-based treatment for multiple sclerosis (MS): a harmonised study protocol for two phase I clinical trials comparing intradermal and intranodal cell administration. *BMJ Open.* (2019) 9:e030309. doi: 10.1136/bmjopen-2019-030309
49. Willekens B, Presas-Rodríguez S, Mansilla M, Derdelinckx J, Lee WP, Nijs G, et al. *Safety and Feasibility of a Tolerogenic Dendritic Cell-Based Treatment of Multiple Sclerosis (MS): A Collaborative Initiative Comparing Intranodal and Intradermal Cell Administration in Two Phase I Clinical Trials*. Stockholm: ECTRIMS Online Library (2019).
50. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med.* (1998) 188:1075–82. doi: 10.1084/jem.188.6.1075
51. Sudowe S, Dominitzki S, Montermann E, Bros M, Grabbe S, Reske-Kunz AB. Uptake and presentation of exogenous antigen and presentation of endogenously produced antigen by skin dendritic cells represent equivalent pathways for the priming of cellular immune responses following biolistic DNA immunization. *Immunology.* (2009) 128(1 Suppl.):e193–205. doi: 10.1111/j.1365-2567.2008.02947.x
52. Lazzaro S, Giovani C, Mangiavacchi S, Magini D, Maione D, Baudner B, et al. CD8 T-cell priming upon mRNA vaccination is restricted to bone-marrow-derived antigen-presenting cells and may involve antigen transfer from myocytes. *Immunology.* (2015) 146:312–26. doi: 10.1111/imm.12505
53. Solvason N, Lou YP, Peters W, Evans E, Martinez J, Ramirez U, et al. Improved efficacy of a tolerizing DNA vaccine for reversal of hyperglycemia through enhancement of gene expression and localization to intracellular sites. *J Immunol.* (2008) 181:8298–307. doi: 10.4049/jimmunol.181.12.8298
54. Waisman A, Ruiz PJ, Hirschberg DL, Gelman A, Oksenberg JR, Brocke S, et al. Suppressive vaccination with DNA encoding a variable region gene of the T-cell receptor prevents autoimmune encephalomyelitis and activates Th2 immunity. *Nat Med.* (1996) 2:899–905. doi: 10.1038/nm0896-899
55. Mbongue JC, Rawson J, Garcia PA, Gonzalez N, Cobb J, Kandeel F, et al. Reversal of new onset type 1 diabetes by oral salmonella-based combination therapy and mediated by regulatory T-cells in NOD mice. *Front Immunol.* (2019) 10:320. doi: 10.3389/fimmu.2019.00320
56. Husseiny MI, Du W, Mbongue J, Lenz A, Rawson J, Kandeel F, et al. Factors affecting Salmonella-based combination immunotherapy for prevention of type 1 diabetes in non-obese diabetic mice. *Vaccine.* (2018) 36:8008–18. doi: 10.1016/j.vaccine.2018.10.101
57. Dou L, Ono Y, Chen YF, Thomson AW, Chen XP. Hepatic dendritic cells, the tolerogenic liver environment, and liver disease. *Semin Liver Dis.* (2018) 38:170–80. doi: 10.1055/s-0038-1646949
58. Keeler GD, Kumar S, Palaschak B, Silverberg EL, Markusic DM, Jones NT, et al. Gene therapy-induced antigen-specific tregs inhibit neuro-inflammation and reverse disease in a mouse model of multiple sclerosis. *Mol Ther.* (2018) 26:173–83. doi: 10.1016/j.ymthe.2017.09.001
59. Han G, Wang R, Chen G, Wang J, Xu R, Feng J, et al. Gene delivery GAD 500 autoantigen by AAV serotype 1 prevented diabetes in NOD mice: transduction efficiency do not play important roles. *Immunol Lett.* (2008) 115:110–6. doi: 10.1016/j.imlet.2007.10.004
60. Akbarpour M, Goudy KS, Cantore A, Russo F, Sanvito F, Naldini L, et al. Insulin B chain 9-23 gene transfer to hepatocytes protects from type 1 diabetes by inducing Ag-specific FoxP3+ Tregs. *Sci Transl Med.* (2015) 7:289ra81. doi: 10.1126/scitranslmed.aaa3032

61. Annoni A, Goudy K, Akbarpour M, Naldini L, Roncarolo MG. Immune responses in liver-directed lentiviral gene therapy. *Transl Res.* (2013) 161:230–40. doi: 10.1016/j.trsl.2012.12.018
62. Pozsgay J, Szekanecz Z, Sarmay G. Antigen-specific immunotherapies in rheumatic diseases. *Nat Rev Rheumatol.* (2017) 13:525–37. doi: 10.1038/nrrheum.2017.107
63. Zhang N, Nandakumar KS. Recent advances in the development of vaccines for chronic inflammatory autoimmune diseases. *Vaccine.* (2018) 36:3208–20. doi: 10.1016/j.vaccine.2018.04.062
64. Bachmann MF, Mohsen MO, Kramer MF, Heath MD. Vaccination against allergy: a paradigm shift? *Trends Mol Med.* (2020) 26:357–68. doi: 10.1016/j.molmed.2020.01.007
65. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, et al. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature.* (2005) 435:220–3. doi: 10.1038/nature03523
66. Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL. Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. *Proc Natl Acad Sci USA.* (1991) 88:10252–6. doi: 10.1073/pnas.88.22.10252
67. Sai P, Rivereau AS. Prevention of diabetes in the nonobese diabetic mouse by oral immunological treatments. Comparative efficiency of human insulin and two bacterial antigens, lipopolysacharide from Escherichia coli and glycoprotein extract from Klebsiella pneumoniae. *Diabetes Metab.* (1996) 22:341–8.
68. Bergerot I, Arreaza GA, Cameron MJ, Burdick MD, Strieter RM, Chensue SW, et al. Insulin B-chain reactive CD4+ regulatory T-cells induced by oral insulin treatment protect from type 1 diabetes by blocking the cytokine secretion and pancreatic infiltration of diabetogenic effector T-cells. *Diabetes.* (1999) 48:1720–9. doi: 10.2337/diabetes.48.9.1720
69. Zhang L, Londono P, Yu L, Grimes S, Blackburn P, Gottlieb P, et al. MAS-1 adjuvant immunotherapy generates robust Th2 type and regulatory immune responses providing long-term protection from diabetes in late-stage pre-diabetic NOD mice. *Autoimmunity.* (2014) 47:341–50. doi: 10.3109/08916934.2014.910768
70. Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol.* (2002) 2:85–95. doi: 10.1038/nri724
71. Yoosuf S, Makharia GK. Evolving therapy for celiac disease. *Front Pediatr.* (2019) 7:193. doi: 10.3389/fped.2019.00193
72. Candia M, Kratzer B, Pickl WF. On peptides and altered peptide ligands: from origin, mode of action and design to clinical application (immunotherapy). *Int Arch Allergy Immunol.* (2016) 170:211–33. doi: 10.1159/000448756
73. Antunes DA, Abella JR, Devaurs D, Rigo MM, Kavraki LE. Structure-based methods for binding mode and binding affinity prediction for peptide-MHC complexes. *Curr Top Med Chem.* (2018) 18:2239–55. doi: 10.2174/1568026619666181224101744
74. Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature.* (1993) 363:156–9. doi: 10.1038/363156a0
75. Pfeiffer C, Stein J, Southwood S, Ketelaar H, Sette A, Bottomly K. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J Exp Med.* (1995) 181:1569–74. doi: 10.1084/jem.181.4.1569
76. Crowe PD, Qin Y, Conlon PJ, Antel JP. NBI-5788, an altered MBP83-99 peptide, induces a T-helper 2-like immune response in multiple sclerosis patients. *Ann Neurol.* (2000) 48:758–65.
77. Banchereau J, Klechevsky E, Schmitt N, Morita R, Palucka K, Ueno H. Harnessing human dendritic cell subsets to design novel vaccines. *Ann N Y Acad Sci.* (2009) 1174:24–32. doi: 10.1111/j.1749-6632.2009.04999.x
78. Gojanovich GS, Murray SL, Buntzman AS, Young EF, Vincent BG, Hess PR. The use of peptide-major-histocompatibility-complex multimers in type 1 diabetes mellitus. *J Diabetes Sci Technol.* (2012) 6:515–24. doi: 10.1177/193229681200600305
79. Spack EG, McCutcheon M, Corbelletta N, Nag B, Passmore D, Sharma SD. Induction of tolerance in experimental autoimmune myasthenia gravis with solubilized MHC class II:acetylcholine receptor peptide complexes. *J Autoimmun.* (1995) 8:787–807. doi: 10.1016/S0896-8411(95)80018-2
80. Vandenbark AA, Rich C, Mooney J, Zamora A, Wang C, Huan J, et al. Recombinant TCR ligand induces tolerance to myelin oligodendrocyte glycoprotein 35-55 peptide and reverses clinical and histological signs of chronic experimental autoimmune encephalomyelitis in HLA-DR2 transgenic mice. *J Immunol.* (2003) 171:127–33. doi: 10.4049/jimmunol.171.1.127
81. Sinha S, Subramanian S, Proctor TM, Kaler LJ, Grafe M, Dahan R, et al. A promising therapeutic approach for multiple sclerosis: recombinant T-cell receptor ligands modulate experimental autoimmune encephalomyelitis by reducing interleukin-17 production and inhibiting migration of encephalitogenic cells into the CNS. *J Neurosci.* (2007) 27:12531–9. doi: 10.1523/JNEUROSCI.3599-07.2007
82. Huan J, Subramanian S, Jones R, Rich C, Link J, Mooney J, et al. Monomeric recombinant TCR ligand reduces relapse rate and severity of experimental autoimmune encephalomyelitis in SJL/J mice through cytokine switch. *J Immunol.* (2004) 172:4556–66. doi: 10.4049/jimmunol.172.7.4556
83. Lin M, Stoica-Nazarov C, Surls J, Kehl M, Bona C, Olsen C, et al. Reversal of type 1 diabetes by a new MHC II-peptide chimera: "Single-epitope-mediated suppression" to stabilize a polyclonal autoimmune T-cell process. *Eur J Immunol.* (2010) 40:2277–88. doi: 10.1002/eji.200940094
84. Getts DR, McCarthy DP, Miller SD. Exploiting apoptosis for therapeutic tolerance induction. *J Immunol.* (2013) 191:5341–6. doi: 10.4049/jimmunol.1302070
85. Cremel M, Guerin N, Horand F, Banz A, Godfrin Y. Red blood cells as innovative antigen carrier to induce specific immune tolerance. *Int J Pharm.* (2013) 443:39–49. doi: 10.1016/j.ijpharm.2012.12.044
86. Kontos S, Kourtis IC, Dane KY, Hubbell JA. Engineering antigens for in situ erythrocyte binding induces T-cell deletion. *Proc Natl Acad Sci USA.* (2013) 110:E60–8. doi: 10.1073/pnas.1216353110
87. Grimm AJ, Kontos S, Diaceri G, Quaglia-Thermes X, Hubbell JA. Memory of tolerance and induction of regulatory T cells by erythrocyte-targeted antigens. *Sci Rep.* (2015) 5:15907. doi: 10.1038/srep15907
88. Pishesha N, Bilate AM, Wibowo MC, Huang NJ, Li Z, Deshycka R, et al. Engineered erythrocytes covalently linked to antigenic peptides can protect against autoimmune disease. *Proc Natl Acad Sci USA.* (2017) 114:3157–62. doi: 10.1073/pnas.1701746114
89. Pujol-Autonell I, Mansilla MJ, Rodriguez-Fernandez S, Cano-Sarabia M, Navarro-Barriuso J, Ampudia RM, et al. Liposome-based immunotherapy against autoimmune diseases: therapeutic effect on multiple sclerosis. *Nanomedicine.* (2017) 12:1231–42. doi: 10.2217/nnm-2016-0410
90. Capini C, Jaturanpinyo M, Chang HI, Mutalik S, McNally A, Street S, et al. Antigen-specific suppression of inflammatory arthritis using liposomes. *J Immunol.* (2009) 182:3556–65. doi: 10.4049/jimmunol.0802972
91. Engman C, Wen Y, Meng WS, Bottino R, Trucco M, Giannoukakis N. Generation of antigen-specific Foxp3+ regulatory T-cells in vivo following administration of diabetes-reversing tolerogenic microspheres does not require provision of antigen in the formulation. *Clin Immunol.* (2015) 160:103–23. doi: 10.1016/j.clim.2015.03.004
92. Phillips B, Nylander K, Harnaha J, Machen J, Lakomy R, Styche A, et al. A microsphere-based vaccine prevents and reverses new-onset autoimmune diabetes. *Diabetes.* (2008) 57:1544–55. doi: 10.2337/db07-0507
93. Pearson RM, Podojil JR, Shea LD, King NJC, Miller SD, Getts DR. Overcoming challenges in treating autoimmuntity: Development of tolerogenic immune-modifying nanoparticles. *Nanomedicine.* (2019) 18:282–91. doi: 10.1016/j.nano.2018.10.001
94. Kishimoto TK, Maldonado RA. Nanoparticles for the induction of antigen-specific immunological tolerance. *Front Immunol.* (2018) 9:230. doi: 10.3389/fimmu.2018.00230
95. Carambia A, Freund B, Schwinge D, Bruns OT, Salmen SC, Ittrich H, et al. Nanoparticle-based autoantigen delivery to Treg-inducing liver sinusoidal endothelial cells enables control of autoimmunity in mice. *J Hepatol.* (2015) 62:1349–56. doi: 10.1016/j.jhep.2015.01.006
96. Clemente-Casares X, Blanco J, Ambalavanan P, Yamanouchi J, Singha S, Fandos C, et al. Expanding antigen-specific regulatory networks to treat autoimmunity. *Nature.* (2016) 530:434–40. doi: 10.1038/nature16962
97. Umeshappa CS, Singha S, Blanco J, Shao K, Nanjundappa RH, Yamanouchi J, et al. Suppression of a broad spectrum of liver autoimmune pathologies by

single peptide-MHC-based nanomedicines. *Nat Commun.* (2019) 10:2150. doi: 10.1038/s41467-019-09893-5
98. Serra P, Santamaria P. Antigen-specific therapeutic approaches for autoimmunity. *Nat Biotechnol.* (2019) 37:238–51. doi: 10.1038/s41587-019-0015-4
99. Gómez López A. Nanotechnology and autoimmunity. In: Rosario UE, editor. *Autoimmunity: From Bench to Bedside.* Bogota: El Rosario University Press. (2013). p. 727–38.
100. Rezaei R, Safaei M, Mozaffari HR, Moradpoor H, Karami S, Golshah A, et al. The role of nanomaterials in the treatment of diseases and their effects on the immune system. *Open Access Maced J Med Sci.* (2019) 7:1884–90. doi: 10.3889/oamjms.2019.486
101. Ten Brinke A, Martinez-Llordella M, Cools N, Hilkens CMU, van Ham SM, Sawitzki B, et al. Ways forward for tolerance-inducing cellular therapies- an AFACTT perspective. *Front Immunol.* (2019) 10:181. doi: 10.3389/fimmu.2019.00181
102. Geissler EK, Hutchinson JA. Cell therapy as a strategy to minimize maintenance immunosuppression in solid organ transplant recipients. *Curr Opin Organ Transplant.* (2013) 18:408–15. doi: 10.1097/MOT.0b013e328363319d
103. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med.* (2004) 199:1455–65. doi: 10.1084/jem.20040139
104. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood.* (2010) 116:935–44. doi: 10.1182/blood-2009-07-234872
105. Mfarrej B, Tresoldi E, Stabilini A, Paganelli A, Caldara R, Secchi A, et al. Generation of donor-specific Tr1 cells to be used after kidney transplantation and definition of the timing of their in vivo infusion in the presence of immunosuppression. *J Transl Med.* (2017) 15:40. doi: 10.1186/s12967-017-1133-8
106. Putnam AL, Safinia N, Medvec A, Laszkowska M, Wray M, Mintz MA, et al. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant.* (2013) 13:3010–20. doi: 10.1111/ajt.12433
107. MacDonald KG, Hoeppli RE, Huang Q, Gillies J, Luciani DS, Orban PC, et al. Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. *J Clin Invest.* (2016) 126:1413–24. doi: 10.1172/JCI82771
108. Boardman DA, Philippeos C, Fruhwirth GO, Ibrahim MA, Hannen RF, Cooper D, et al. Expression of a chimeric antigen receptor specific for donor HLA Class I enhances the potency of human regulatory T cells in preventing human skin transplant rejection. *Am J Transplant.* (2017) 17:931–43. doi: 10.1111/ajt.14185
109. Dwyer CJ, Ward NC, Pugliese A, Malek TR. Promoting immune regulation in type 1 diabetes using low-dose interleukin-2. *Curr Diab Rep.* (2016) 16:46. doi: 10.1007/s11892-016-0739-1
110. Tang Q, Vincenti F. Transplant trials with Tregs: perils and promises. *J Clin Invest.* (2017) 127:2505–12. doi: 10.1172/JCI90598
111. Gregori S. Dendritic cells in networks of immunological tolerance. *Tissue Antigens.* (2011) 77:89–99. doi: 10.1111/j.1399-0039.2010.01615.x
112. Osorio F, Fuentes C, Lopez MN, Salazar-Onfray F, Gonzalez FE. Role of dendritic cells in the induction of lymphocyte tolerance. *Front Immunol.* (2015) 6:535. doi: 10.3389/fimmu.2015.00535
113. Raker VK, Domogalla MP, Steinbrink K. Tolerogenic dendritic cells for regulatory T cell induction in man. *Front Immunol.* (2015) 6:569. doi: 10.3389/fimmu.2015.00569
114. Liu J, Cao X. Regulatory dendritic cells in autoimmunity: a comprehensive review. *J Autoimmun.* (2015) 63:1–12. doi: 10.1016/j.jaut.2015.07.011
115. Ten Brinke A, Hilkens CM, Cools N, Geissler EK, Hutchinson JA, Lombardi G, et al. Clinical use of tolerogenic dendritic cells-harmonization approach in European Collaborative Effort. *Mediators Inflamm.* (2015) 2015:471719. doi: 10.1155/2015/471719
116. Amodio G, Cichy J, Conde P, Matteoli G, Moreau A, Ochando J, et al. Role of myeloid regulatory cells (MRCs) in maintaining tissue homeostasis and promoting tolerance in autoimmunity, inflammatory disease and transplantation. *Cancer Immunol Immunother.* (2019) 68:661–72. doi: 10.1007/s00262-018-2264-3
117. Obregon C, Kumar R, Pascual MA, Vassalli G, Golshayan D. Update on dendritic cell-induced immunological and clinical tolerance. *Front Immunol.* (2017) 8:1514. doi: 10.3389/fimmu.2017.01514
118. Amodio G, Comi M, Tomasoni D, Gianolini ME, Rizzo R, Lemaoult J, et al. Hla-g expression levels influence the tolerogenic activity of human DC-10. *Haematologica.* (2015) 100:548–57. doi: 10.3324/haematol.2014.113803
119. Mellor AL, Lemos H, Huang L. Indoleamine 2,3-Dioxygenase and tolerance: where are we now? *Front Immunol.* (2017) 8:1360. doi: 10.3389/fimmu.2017.01360
120. Spiering R, Jansen MAA, Wood MJ, Fath AA, Eltherington O, Anderson AE, et al. Targeting of tolerogenic dendritic cells to heat-shock proteins in inflammatory arthritis. *J Transl Med.* (2019) 17:375. doi: 10.1186/s12967-019-2128-4
121. Domogalla MP, Rostan PV, Raker VK, Steinbrink K. Tolerance through education: how tolerogenic dendritic cells shape immunity. *Front Immunol.* (2017) 8:1764. doi: 10.3389/fimmu.2017.01764
122. Wang Y, Sosinowski T, Novikov A, Crawford F, Neau DB, Yang J, et al. C-terminal modification of the insulin B:11-23 peptide creates superagonists in mouse and human type 1 diabetes. *Proc Natl Acad Sci USA.* (2018) 115:162–7. doi: 10.1073/pnas.1716527115
123. Delong T, Wiles TA, Baker RL, Bradley B, Barbour G, Reisdorph R, et al. Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. *Science.* (2016) 351:711–4. doi: 10.1126/science.aad2791
124. Kracht MJ, van Lummel M, Nikolic T, Joosten AM, Laban S, van der Slik AR, et al. Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes. *Nat Med.* (2017) 23:501–7. doi: 10.1038/nm.4289

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 02 October 2020
doi: 10.3389/fimmu.2020.536326

The Regulatory Function of CCR9$^+$ Dendritic Cells in Inflammation and Autoimmunity

Manisha Pathak and Girdhari Lal *

Laboratory of Autoimmunity and Tolerance, National Centre for Cell Science, Pune, India

Chemokine receptor CCR9 is a G protein–coupled receptor and expressed on several types of immune cells, including dendritic cells (DCs), CD4$^+$ T cells, and B cells. CCR9 drives the migration of immune cells to gradients of its cognate ligand CCL25. The chemokine CCL25 is mostly produced by gut and thymic epithelial cells. Gut- and thymic-homing DCs are known to express CCR9, and these cells are predominantly localized in the gut lining and thymus. CCR9$^+$ DCs are implicated in regulating inflammation, food allergy, alloimmunity, and autoimmunity. Differential interaction of CCR9$^+$ DCs with lymphoid and myeloid cells in the thymus, secondary lymphoid tissues, and mucosal sites offer crucial insights to immune regulation. In this review, we examine the phenotypes, distributions, and interactions of CCR9$^+$ DCs with other immune cells, elucidating their functions and role in inflammation and autoimmunity.

Keywords: C-C chemokine receptor type 9 (CCR9), Foxp3$^+$ Regulatory T cells, CCL25, dendritic cell (DC), mucosal tolerance

OPEN ACCESS

Edited by:
Femke Broere,
Utrecht University, Netherlands

Reviewed by:
Magali Irla,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Iain Comerford,
University of Adelaide, Australia

***Correspondence:**
Girdhari Lal
glal@nccs.res.in

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *19 February 2020*
Accepted: *13 August 2020*
Published: *02 October 2020*

Citation:
Pathak M and Lal G (2020) The Regulatory Function of CCR9$^+$ Dendritic Cells in Inflammation and Autoimmunity. Front. Immunol. 11:536326. doi: 10.3389/fimmu.2020.536326

INTRODUCTION

Chemokine receptor CCR9 has important homeostatic and regulatory functions and drives the migration of immune cells to the gradient of CCL25 (also known as thymus-expressed chemokine, TECK). Expression of CCR9 is reported on the majority of gut-homing CD4$^+$ and CD8$^+$ T cells, gamma-delta T cells, plasmacytoid dendritic cells (pDCs), IgA plasmablast, IgA plasma cells, and intraepithelial lymphocytes (IELs) (1–3). Thymic and intestinal epithelial cells constitutively express CCL25 in mice and humans (4), and it is overexpressed in the intestine during gut inflammation and autoimmunity (1, 5–9). Due to the upregulation of CCL25 and recruitment of CCR9$^+$ immune cells in the gut of inflammatory bowel disease (IBD) patients, CCR9 is considered as a potential therapeutic target to control gut inflammation (1, 10, 11). However, Ccr9$^{-/-}$ or Ccl25$^{-/-}$ mice show increased severity of dextran sodium sulfate (DSS)-induced colitis (12). Ccr9$^{-/-}$ mice have very low capacity to induce immune tolerance to oral antigens (5). In parallel, clinical trials with CCR9 antagonist CCX282-B have been disappointing and display dose-dependent adverse reactions in Crohn's disease (10, 11, 13). Further, it is shown that CCR9 expression in CD4$^+$ T cells in gut inflammation has a dispensable function (14).

On the other hand, DCs play an important role in maintaining gut homeostasis and inflammation in mice and humans. DCs in mouse lymphoid organs can be broadly classified into two groups: conventional DCs (cDC; B220$^-$CD11c^{hi}) and pDCs (B220$^+$CD11c^{int}). Using flow cytometry and CyTOF, multidimensional analysis of DCs in the different tissues in humans and

mice is been elegantly characterized and shows a heterogeneous population of DCs than just cDCs and pDCs (15). A study in 2009 by a group of researchers led by Villandangos show that both DC subsets differ in their developmental and functional properties (16). The DCs are known to exhibit inflammatory and tolerogenic functions in the gut. Gut DCs are present within the gut-associated lymphoid tissues (GALTs), which include Peyer's patch (PP) and solitary isolated lymphoid tissues (ILT) or even distributed throughout lamina propria (LP) (17). Gut-homing DCs are further classified into four subsets based on the expression of the surface markers CD103 and CD11b; $CD103^+CD11b^-$, $CD103^-CD11b^+$, $CD103^+CD11b^+$ and $CD103^-CD11b^-$ (18). $CD103^+CD11b^-$ falls under cDC1, whereas $CD103^+CD11b^+$ forms subgroup cDC2 (15, 19, 20). CCR9 and other gut-homing chemokine receptors drive the migration of DCs into the GALTs. The localization of DCs in secondary lymphoid tissues, and its interaction with immune cells meticulously tunes the balance between homeostasis and inflammation. In this review, we discuss how $CCR9^+$ DCs regulate the phenotype and function of innate and adaptive immune cells during homeostasis and tolerance.

DISTRIBUTION AND FUNCTION OF $CCR9^+$ DCs

In the gut, DCs are present in mesenteric lymph node (mLN), LP, and PP to mount an effective immune response (21, 22). The mucosal tolerance induction by DCs to innocuous antigens by promoting Tregs and producing sIgA in the intestine are discussed earlier (22). However, during inflammation, DCs promote the generation of Th1/Th17 by secreting proinflammatory cytokines (23). CCR9 controls the migration of $CD11c^+$ DCs into the gut (14, 24) and also drives the recruitment of various subsets of DCs into the PP and mLN during inflammation (14). PP with specialized M cells uptake and recognize particulate antigens in a controlled manner and induce tolerance during homeostasis (25, 26). In colitis, many DCs are recruited into the subepithelial dome. These DCs internalize the bacteria and translocate into the PP (27). $CD103^+$ DCs play a protective role at the initial phase of inflammation, whereas in the chronic phase, $CD11b^+$ DCs show a pathogenic role by inducing Th1/Th17 response (28, 29). Our recent study demonstrates that CCR9 is expressed in various subsets of DCs during homeostatic and gut inflammation (14). Inflammation increases CCR9 expression on $CD103^+CD11b^-$, $CD103^-CD11b^+$, and $CD103^+CD11b^+$ subset in PP and mLN (14). Thus, CCR9 affects the distribution of a different subset of cDCs in the gut during homeostasis and inflammation (**Figure 1**). A study from Wendland et al. reveals that CCR9 controls the migration of pDCs into the gut under homeostasis and inflammation. Furthermore, these intestinal pDCs help in the rapid mobilization of myeloid DCs into LP (8). In addition to distribution, CCR9 affects the function of DCs during inflammation (14, 24). Previous work has shown that $CCR9^+$ pDCs inhibit T cell proliferation and induce $Foxp3^+$ regulatory T cells (7). However, the intrinsic mechanism of CCR9 signaling that controls the expression of costimulatory and regulatory molecules on cDCs and its effects on the distribution and function of DCs in the GALTs require detailed investigation.

ROLE OF $CCR9^+$ DCs IN THE INDUCTION OF CENTRAL TOLERANCE

The role of DCs in inducing peripheral and central tolerance is well known (7, 30, 31). In thymus, DCs have extrathymic and intrathymic origins and are very heterogeneous. In the thymic medulla, DCs and medullary thymic epithelial cells (mTEC) express MHC-I and MHC-II and act as very important antigen-presenting cells. The negative selection of αβ TCR cells and $Foxp3^+$ Treg development requires direct recognition of self-antigens *via* MHC class II present on mTEC and thymic DCs (32–34). DCs can take up antigens from the peripheral tissues and migrate into the thymus, thus playing a role in controlling the development of $Foxp3^+$ natural Tregs (nTregs) (35). CCR2 and CCR9 are crucial chemokine receptors involved in the homing of DCs in the thymus (31, 36). CCR9 is expressed at an early developmental T cell stage (double negative 3; DN3 stage), during which thymocytes undergo β selection (rearranging of the TCR beta chain expression along with the pre-T alpha chain) (37). The successful β selection leads the thymocytes to enter the DN4 stage and become $CD4^+CD8^+$ thymocytes and then further undergo positive and negative selection. In the thymic microenvironment, thymic stromal cells express chemokine CCL25 and CCL2 and control the migration of thymic DC and control the central tolerance (4, 36). Thymic cDC2 expressing CCR2 and $Ccr2^{-/-}$ mice show defective negative selection (38). pDC in the thymus expressing CCR9 and $Ccr9^{-/-}$ mice show a defect in the migration of pDC in the thymus as well as impairment in thymocyte deletion (31). It has been reported that CCR7 drives the recruitment of cDCs in the thymus as $Ccr7^{-/-}$, $Ccl21a^{-/-}$, or $Ccl19^{-/-}$ mice that show a defect in the migration of cDC progenitors (39). CCL2/CCR2 interaction helps in the migration of cDCs into the thymic cortex and localizing them to perivascular spaces where they further participate in central tolerance by depleting autoreactive T cell clones (36, 38, 40). This homing process is also controlled by lymphotoxin α (LTα), which negatively regulates CCL2, CCL8, and CCL12 chemokines in the thymus (40). CCL8 is also a ligand for CCR1 and CCR5 and involved in the migration of pDCs and cDCs in the thymus (40). Our recent study also suggests that $CD103^+$ DCs and thymic DCs are a potent inducer of Treg in the presence of CCL25 (14). Thus, chemokine receptors play an important role in the thymic settling of DCs and controlling the central tolerance.

MOLECULAR MECHANISM OF $CCR9^+$ DCs IN INFLAMMATION AND AUTOIMMUNITY

Upon antigen encounter, various signaling pathways, such as JAK/STAT3, Wnt/β-catenin, and AKT/mTOR pathways, get

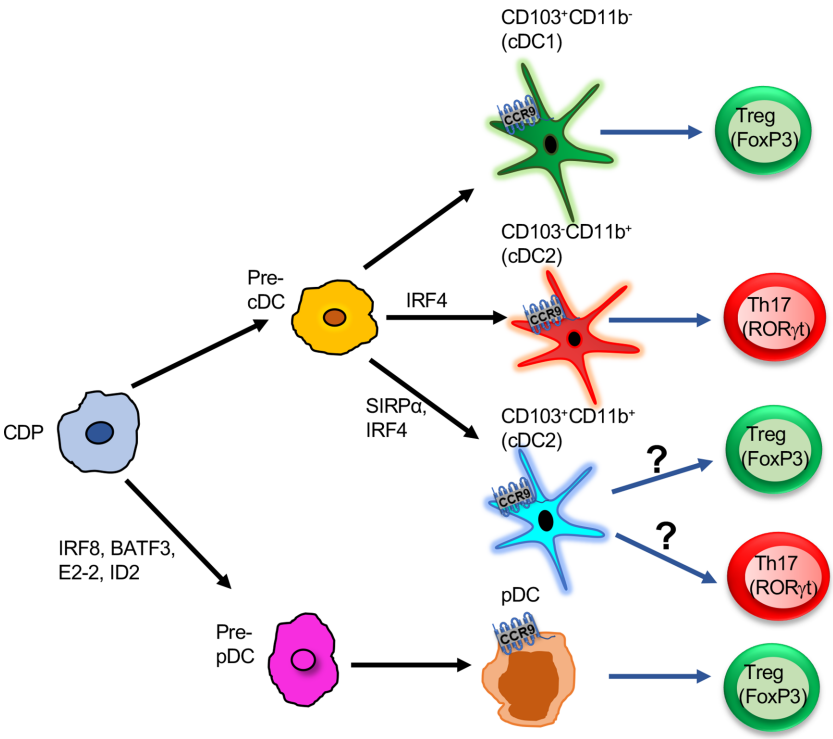


FIGURE 1 | Functions of CCR9+ DCs in the intestine: Pre-pDCs or pre-cDCs derived from CDP progenitors. Further pre-cDCs are differentiated into three subsets based on the expression of surface markers CD103 and CD11b: $CD103^{+}CD11b^{-}$, $CD103^{-}CD11b^{+}$, $CD103^{+}CD11b^{+}$. All three subsets express CCR9 and induce either T cells to differentiate into Tregs or Th17. $CD103^{+}CD11b^{-}$ is the potent inducer of Treg cells. Pre-pDCs differentiate into pDCs, and CCR9+ pDCs promote Treg differentiation in the gut. CDP, Common dendritic cell progenitor; Pre-cDC, pre-common dendritic cell; Pre-pDC, pre-plasmacytoid dendritic cell; cDC, common dendritic cell; Treg, T regulatory cells; pDC, plasmacytoid dendritic cell.

activated in DCs, altering gene expression (41). STAT3 and MAP kinase signaling activate IL-10, TGF-β, and aldehyde dehydrogenase (ALDH), which, in turn, induces tolerance, and any disruption in these pathways leads to loss of T cell tolerance and cause gut inflammation (41–43). However, little is known about the molecular mechanism of how CCR9 affects DC function and their phenotype. Only a few studies have attempted to address this issue and provide preliminary insights into the underlying molecular mechanisms in DCs. DCs are well-known antigen-presenting cells, and CCR9 signaling DCs has an inverse relation in DC maturation (24). V-ATPase is known to play an important role in homeostasis and disease. V-ATPases are affected by activation through toll-like receptor signaling, glucose, and amino acid availability in the microenvironment (44). PI-3 kinase and mTOR signaling is known to upregulate DC maturation by assembling the domains of V-ATPase. Consequentially, inhibition of the V-ATPase domain assembly may affect the antigen processing and presentation in the DCs and promoting tolerogenic phenotype (45). Previous studies report that the NFκB pathway prevents the transcription of proinflammatory genes and promotes the tolerogenic DC phenotype. NFκB is designated as a critical marker of TSLP production in airway epithelial cells (46, 47). As a prelude to unraveling the functional and phenotypic insights under the homeostasis condition, we show that $CCR9^{hi}$ DCs have a lower costimulatory molecule and show thymic-stromal lymphopoietin (TSLP)-mediated regulation of the immune response (14). Based on these recent investigations, we suggest that it could be possible that CCR9 may use this mechanism to prevent the maturation of DCs by inhibiting the V-ATPase domain assembly or activating the NFκB pathway to regulate TSLP secretion. In either case, it may contribute to the tolerogenic function of DCs (45, 46). Thus, we comment that, apart from these two possible mechanisms, CCR9 may regulate other signaling pathways in DCs, contributing to tolerogenic function in the gut, which is an interesting area of research requiring further investigation. In the next sections, we critically review the potential role of CCR9+ DCs in regulating the $CD4^{+}$ T cell, B cell, and innate lymphoid cell responses.

Regulation of Different Subsets of CD4 T Cells

As discussed above, inflammation induces the expression of CCL25 to several folds in intestinal epithelial cells and drives the recruitment of various subsets of DCs (14, 48). Apart from the chemotactic role of CCR9 to CCL25, we follow up in our recent study to show how its intrinsic signaling in DCs affects the differentiation of regulatory $Foxp3^{+}$ $CD4^{+}$ T cells (Tregs) (14).

We recall that CCR9$^+$ DCs are present in the mLN and PP, promoting Tregs' differentiation, whereas CCR9$^-$ DCs drive the Th17 cells in the presence of CCL25 (14). These studies indicate that CCR9$^-$ DCs are more inflammatory in the phenotype and drive the differentiation of naïve CD4$^+$ T cells into Th1/Th17. Intriguingly, CCR9$^+$ DCs show reduced expression of costimulatory molecules (MHC II and CD86) and increased expression of regulatory molecules such as FasL and latency-associated peptides (LAP) (14, 49, 50). A study from the Blanchard group in 2009 shows that higher expression of CCR9 inhibits IL-2 production, causing apoptosis of T cells and promoting tolerance in mice (24). In contrast, the low or absent CCR9 on bone marrow (BM)–generated DCs in the presence of GM-CSF increases the expression of inflammatory molecules, which, in turn, induces proliferation and expansion of T cells (24). However, it is reported that BM treated with GM-CSF gives rise to macrophages (51). A study from Wurbel et al. shows that the CCR9$^+$ macrophage responds to CCL25 gradient and displays proinflammatory and anti-inflammatory functions (48). Therefore, further studies are required to understand how CCL25/CCR9 interaction regulates macrophage function. The different subsets of DCs show diverse functions in the intestine by expressing various surface molecules and cytokines (41). CD103$^+$ DCs mediate tolerogenic function (52) while CD11b$^+$ DCs regulate inflammatory responses by producing IL-12, IL-23, iNOS, and TNF-α (53). Our recent study shows that CD103$^+$CD11b$^-$ DCs promote Treg differentiation in the presence of CCL25 (14). TSLP is highly expressed by CD103$^+$ DCs, which promote the differentiation of Foxp3$^+$ Tregs by directly interacting with its receptor on CD4$^+$ T cells or limiting their potential to drive Th1 cells (54). These studies suggest that CCR9$^+$CD103$^+$ DCs are the most probable promoters of Treg induction *via* secreting TSLP molecule while CCR9$^+$CD11b$^+$ DCs induce the Th1/Th17 response by expressing proinflammatory cytokines (14, 20, 55). We comment that it could be possible that, during inflammation, CD11b$^+$ DCs lose CCR9 expression due to altered gene expression and promoting proinflammatory response. Nonetheless, the role of TSLP in the presence or absence of CCL25 in DCs require further investigation.

Regulation of B Cell Response

The incoming antigens into the GALTs are sampled by DCs that reside just beneath the subepithelial dome (SED) region underlying the follicle-associated epithelium (FAE) (25). This local sampling of antigens by DCs in the PP established by studies so far is believed to be critical to the induction of adaptive mucosal immunity (56, 57). On the other hand, IgA class switching occurs in both a T cell–dependent and –independent manner (58). Tolerogenic DCs, therefore, trigger the inductive and effector phase of the IgA response in a T cell–dependent route in the PP (57, 58). DCs are known to offer antigens to CD4$^+$ T cells in the perifollicular region of PP or B cell in the SED, which, in turn, activates the TGF-β pathway and promotes IgA class switching and generates high-affinity IgA antibodies (57). These DCs further help in the migration of the plasma cell precursor to LP by upregulating the expression of gut-homing receptors, α4β7-integrin and CCR9 (59). In the T cell–independent pathway, epithelial cells trigger DCs to increase the expression of both B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), which promotes IgA class switching (60). TSLP also provides an autocrine effect on DC and increases expression of BAFF or APRIL, which is required for IgA class switching in the intestine (**Figure 2**). In addition, BAFF and APRIL are also critical regulators of the IgE-specific class-switch recombination (CSR) in the presence of IL-4 (61). On the other hand, our study elucidates that the adoptive transfer of CCR9$^+$ DCs in an ova-allergy model reduces the IgE response (14) and marginally increases IgA$^+$ B cells in the PP and mLN. The presence of cytokines other than TGF-β is known to induce IgG or IgE class switching over the IgA class. With our recent studies in hand, we hypothesize one alternative to the above previously proposed mechanism, i.e., CCR9$^+$ DC inhibits IL-4 production, which activates B cells toward IgA switching over to IgE. However, further mechanistic details of how CCR9$^+$ DCs regulate B cell class switching needs allied investigation and is currently beyond the scope of this review.

Regulation of Innate Immune Cells

From the preceding sections, it is clear that the role of CCR9$^+$ DCs in regulating innate immune cell distribution and function in the intestine currently suffers from poor characterization. Two independent research teams led by Pizzaro and Artisin 2015 in concert show that DCs and innate lymphoid cells (ILCs) cross-talk with each other to maintain gut tolerance, and any perturbation in this cross-talk leads to gut inflammation and colitis (62, 63). On the other hand, ILCs are divided into three subsets. ILC1 is regulated by the transcription factor T-bet and produces cytokines IFN-γ and TNF-α (64). The second group, ILC2, is controlled by transcription factors GATA-3 and Bcl11b and produces Th2 cytokines (65, 66). The third group, ILC3, depends on transcription factor RORγt, and secretes IL-17 and IL-22 cytokines (67). ILC1 and ILC3 play pathogenic roles and are implicated in the epithelial and LP compartment of a mouse model of IBD (62, 63). It is shown that ILC1 is increased in patients with Crohn's disease (CD), whereas ILC2 is increased in patients with ulcerative colitis (UC) (68). However, IBD patients with established UC and CD have increased frequency of ILC1 and ILC2 (68). It is shown that functional cross-talk between human DCs and ILCs occurs across the lymphoid and nonlymphoid (mucosal) tissues (69). DCs regulate the function of ILCs by producing various cytokines, such as IL-23 and IL-1β (62, 70). TLR5 activation on DCs augments IL-23 production, which induces ILC3 to produce cytokine IL-22 (70). Consequentially, IL-22 helps in maintaining epithelial cell integrity by inducing the production of antimicrobial peptides (AMPs), such as regenerating islet-derived protein 3 beta (RegIII-β) and RegIII-γ and mucins from epithelial cells (71). The absence of IL-22 increases Th17 cell expansion and promotes colitis in mice (71). In contrast, ILC3 also regulates IL-22 production by activating lymphotoxin (LT) signaling, which contributes to the development of lymphoid follicles

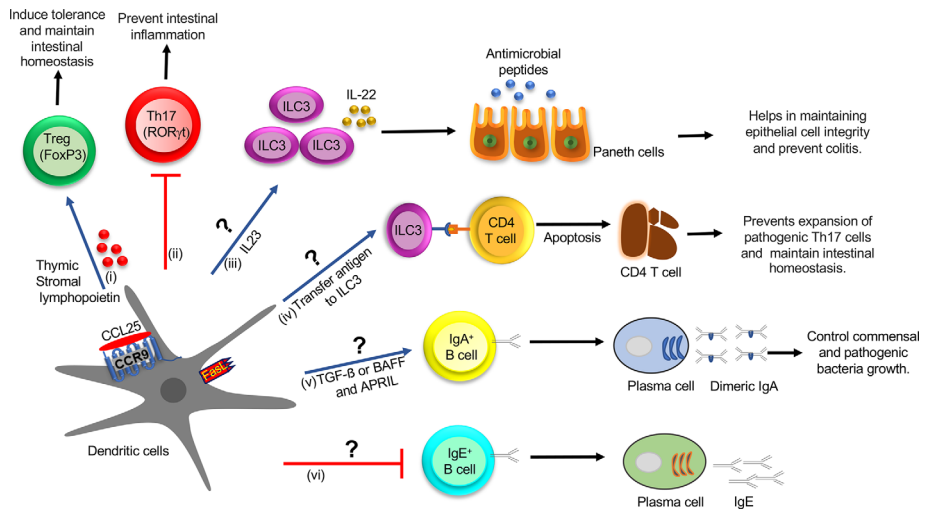


FIGURE 2 | Role of CCR9$^+$ DCs in the regulation of innate and adaptive immune cell function in the intestine during homeostasis: (i) CCL25-CCR9 interaction on DCs increase the production of TSLP and expression of FasL and LAP, which promotes Treg differentiation, which induces tolerance and maintains intestinal homeostasis. (ii) CCR9$^+$ DCs inhibit Th17 differentiation by an unknown mechanism, which prevents intestinal inflammation. (iii) CCR9$^+$ DCs might regulate the ILC3 function by regulating IL-22 production by secreting IL-23 cytokine. IL-22 augments the production of AMP and helps in maintaining epithelial cell integrity and preventing colitis. (iv) ILC3 may acquire antigens from CCR9$^+$ DCs and eliminate commensal-reactive CD4$^+$ T cells by activating the apoptotic pathway, which prevents the expansion of pathogenic Th17 cells and maintains intestinal homeostasis. (v) CCR9$^+$ DCs may regulate IgA$^+$ B cell class switching by activating the TGF-β pathway or increasing expression of BAFF or APRIL and generate high-affinity IgA antibodies, which control commensal and pathogenic bacteria growth. TSLP provides an autocrine effect on DCs and increases expression of BAFF or APRIL, which help in IgA class switching. (vi) CCR9$^+$ DCs may inhibit IgE class switching by an unknown mechanism. DC, Dendritic cell; Treg, T regulatory cells; TSLP, thymic stromal lymphopoietin; LAP, latency-associated peptide; ILCs, innate lymphoid cells; AMP, antimicrobial peptide; BAFF, B-cell activating factor; APRIL, A proliferation-inducing ligand.

(LF) in the gut (72). In the LF, the interaction of ILC3 and DCs through lymphotoxin beta receptor (LTβR) signaling controls the IL-22 synthesis in ILCs (72). In addition, IL-1β regulates the release of Csf2 by ILC3, which promotes the secretion of retinoic acid (RA) and IL-10 from DCs and macrophages to generate homeostasis in the gut (73). ILC3 acquires antigen from CD103$^+$ DCs in LP and eliminates commensal-reactive CD4$^+$ T cells in the mLN during homeostasis (74). ILC3 T cell interaction inhibits IL-2 production and induces apoptosis of effector CD4$^+$ T cells (74). However, in IBD, this function is compromised due to the low expression of MHC-II on ILC3, which governs the expansion of pathogenic Th17 cells (74). Hepworth et al. shows that ILC3 and thymic epithelial cells show regulation of MHC-II expression, and MHC-II$^+$ ILC3s can directly induce cell death in activated commensal bacteria-specific CD4$^+$ T cells (75). Together, these studies indicate that CCR9$^+$CD103$^+$ DCs may induce suppressor function in T cells directly by secreting TSLP and indirectly *via* regulating MHC-II expression on ILC3 and during inflammation. We suggest that ILC3 may acquire antigens from other subsets of CCR9$^+$ DC, which perturbs the MHC-II presentation of ILC3 and induces a Th17 response. In conclusion, ILCs are crucial determinants of pathogen immunity and intestinal homeostasis. Nonetheless, the mechanism of CCR9$^+$ DC regulation by ILCs in the intestine during colitis remains a stone unturned and requires further investigation.

FUTURE PERSPECTIVES

Our recent study in the mouse model shows that CCR9$^+$ DCs contribute to controlling the intestinal inflammation by regulating innate and adaptive immune responses (14). CCL25-CCR9 is studied mostly as a homing receptor. Like another gut-tropic chemokine receptor CCR6 intrinsic signaling, known to alter the phenotype and function of CD4 T cells (76), how the intrinsic signaling of CCR9 manipulates the phenotype and function of DCs is not known. In this review, we focus on the inevitable role of CCR9 in the migration of DCs and how it affects its function during gut inflammation. This review spells out that further studies are indispensable to define intrinsic molecular and cellular signaling of CCR9 in various subsets of DCs. Such studies are expected to offer new pathways to control intestinal inflammation and autoimmunity. Future studies with specific deletion of CCR9 in the subsets of DCs during intestinal inflammation will throw more light on its importance under both homeostatic and inflammatory conditions.

AUTHOR CONTRIBUTIONS

MP and GL wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

MP received the Research Associate fellowship from Science and Engineering Research Board, Ministry of Science and Technology, Government of India (PDF/2015/000792). GL received grants from the Department of Biotechnology, (Grants numbers, BT/PR15533/MED/30/1616/2015; BT/PR14156/BRB/10/1515/2016), Swarnjayanti Fellowship (DST/SJF/LSA-01/2017-18) from Department of Science and Technology, Ministry of Science and Technology, Government of India.

REFERENCES

1. Kulkarni N, Pathak M, Lal G. Role of Chemokine Receptors and Intestinal Epithelial Cells in the Mucosal Inflammation and Tolerance. *J Leukoc Biol* (2017) 101(2):377–94. doi: 10.1189/Jlb.1RU0716-327R
2. Chistiakov DA, Orekhov AN, Sobenin IA, Bobryshev YV. Plasmacytoid dendritic cells: development, functions, and role in atherosclerotic inflammation. *Front Physiol* (2014) 5:279. doi: 10.3389/fphys.2014.00279
3. Wurbel MA, Malissen M, Guy-Grand D, Meffre E, Nussenzweig MC, Richelme M, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. *Blood* (2001) 98 (9):2626–32. doi: 10.1182/blood.v98.9.2626
4. Wurbel MA, Philippe JM, Nguyen C, Victorero G, Freeman T, Wooding P, et al. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. *Eur J Immunol* (2000) 30(1):262–71. doi: 10.1002/1521-4141(200001)30:1<262::AID-IMMU262>3.0.CO;2-0
5. Cassani B, Villablanca EJ, Quintana FJ, Love PE, Lacy-Hulbert A, Blaner WS, et al. Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology* (2011) 141(6):2109–18. doi: 10.1053/j.gastro.2011.09.015
6. Pabst O, Ohl L, Wendland M, Wurbel MA, Kremmer E, Malissen B, et al. Chemokine receptor CCR9 contributes to the localization of plasma cells to the small intestine. *J Exp Med* (2004) 199(3):411–6. doi: 10.1084/jem.20030996
7. Hadeiba H, Sato T, Habtezion A, Oderup C, Pan J, Butcher EC. CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. *Nat Immunol* (2008) 9(11):1253–60. doi: 10.1038/ni.1658
8. Wendland M, Czeloth N, Mach N, Malissen B, Kremmer E, Pabst O, et al. CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine. *Proc Natl Acad Sci U S A* (2007) 104(15):6347–52. doi: 10.1073/pnas.0609180104
9. Uehara S, Grinberg A, Farber JM, Love PE. A role for CCR9 in T lymphocyte development and migration. *J Immunol* (2002) 168(6):2811–9. doi: 10.4049/jimmunol.168.6.2811
10. Wendt E, Keshav S. CCR9 antagonism: potential in the treatment of Inflammatory Bowel Disease. *Clin Exp Gastroenterol* (2015) 8:119–30. doi: 10.2147/CEG.S48305
11. Keshav S, Vanasek T, Niv Y, Petryka R, Howaldt S, Bafutto M, et al. A randomized controlled trial of the efficacy and safety of CCX282-B, an orally-administered blocker of chemokine receptor CCR9, for patients with Crohn's disease. *PloS One* (2013) 8(3):e60094. doi: 10.1371/journal.pone.0060094
12. Wurbel MA, McIntire MG, Dwyer P, Fiebiger E. CCL25/CCR9 interactions regulate large intestinal inflammation in a murine model of acute colitis. *PloS One* (2011) 6(1):e16442. doi: 10.1371/journal.pone.0016442
13. Feagan BG, Sandborn WJ, D'Haens G, Lee SD, Allez M, Fedorak RN, et al. Randomised clinical trial: vercirnon, an oral CCR9 antagonist, vs. placebo as induction therapy in active Crohn's disease. *Alimentary Pharmacol Ther* (2015) 42(10):1170–81. doi: 10.1111/apt.13398
14. Pathak M, Padghan P, Halder N, Shilpi, Kulkarni N, Sonar SA, et al. CCR9 signaling in dendritic cells drives the differentiation of Foxp3(+) Tregs and suppresses the allergic IgE response in the gut. *Eur J Immunol* (2020) 50 (3):404–17. doi: 10.1002/eji.201948327
15. Guilliams M, Dutertre CA, Scott CL, McGovern N, Sichien D, Chakarov S, et al. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity* (2016) 45(3):669–84. doi: 10.1016/j.immuni.2016.08.015
16. Segura E, Wong J, Villadangos JA. Cutting edge: B220+CCR9- dendritic cells are not plasmacytoid dendritic cells but are precursors of conventional dendritic cells. *J Immunol* (2009) 183(3):1514–7. doi: 10.4049/jimmunol.0901524
17. Bekiaris V, Persson EK, Agace WW. Intestinal dendritic cells in the regulation of mucosal immunity. *Immunol Rev* (2014) 260(1):86–101. doi: 10.1111/imr.12194
18. Persson EK, Scott CL, Mowat AM, Agace WW. Dendritic cell subsets in the intestinal lamina propria: ontogeny and function. *Eur J Immunol* (2013) 43 (12):3098–107. doi: 10.1002/eji.201343740
19. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med* (2009) 206(13):3115–30. doi: 10.1084/jem.20091756
20. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* (2013) 38(5):970–83. doi: 10.1016/j.immuni.2013.04.011
21. Chang SY, Ko HJ, Kweon MN. Mucosal dendritic cells shape mucosal immunity. *Exp Mol Med* (2014) 46:e84. doi: 10.1038/emm.2014.16
22. Iwasaki A. Mucosal dendritic cells. *Annu Rev Immunol* (2007) 25:381–418. doi: 10.1146/annurev.immunol.25.022106.141634
23. Niess JH. Role of mucosal dendritic cells in inflammatory bowel disease. *World J Gastroenterol* (2008) 14(33):5138–48. doi: 10.3748/wjg.14.5138
24. Drakes ML, Stiff PJ, Blanchard TG. Inverse relationship between dendritic cell CCR9 expression and maturation state. *Immunology* (2009) 127(4):466–76. doi: 10.1111/j.1365-2567.2009.03043.x
25. Reboldi A, Cyster JG. Peyer's patches: organizing B-cell responses at the intestinal frontier. *Immunol Rev* (2016) 271(1):230–45. doi: 10.1111/imr.12400
26. Lelouard H, Fallet M, de Bovis B, Meresse S, Gorvel JP. Peyer's patch dendritic cells sample antigens by extending dendrites through M cell-specific transcellular pores. *Gastroenterology* (2012) 142(3):592–601.e3. doi: 10.1053/j.gastro.2011.11.039
27. Salim SY, Silva MA, Keita AV, Larsson M, Andersson P, Magnusson KE, et al. CD83+CCR7- dendritic cells accumulate in the subepithelial dome and internalize translocated Escherichia coli HB101 in the Peyer's patches of ileal Crohn's disease. *Am J Pathol* (2009) 174(1):82–90. doi: 10.2353/ajpath.2009.080273
28. Abe K, Nguyen KP, Fine SD, Mo JH, Shen C, Shenouda S, et al. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. *Proc Natl Acad Sci U S A* (2007) 104(43):17022–7. doi: 10.1073/pnas.0708469104
29. Rutella S, Locatelli F. Intestinal dendritic cells in the pathogenesis of inflammatory bowel disease. *World J Gastroenterol* (2011) 17(33):3761–75. doi: 10.3748/wjg.v17.i33.3761
30. Oh J, Shin JS. The Role of Dendritic Cells in Central Tolerance. *Immune Netw* (2015) 15(3):111–20. doi: 10.4110/in.2015.15.3.111
31. Hadeiba H, Lahl K, Edalati A, Oderup C, Habtezion A, Pachynski R, et al. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. *Immunity* (2012) 36(3):438–50. doi: 10.1016/j.immuni.2012.01.017
32. Gallegos AM, Bevan MJ. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med* (2004) 200(8):1039–49. doi: 10.1084/jem.20041457
33. Herbin O, Bonito AJ, Jeong S, Weinstein EG, Rahman AH, Xiong H, et al. Medullary thymic epithelial cells and CD8alpha+ dendritic cells coordinately regulate central tolerance but CD8alpha+ cells are dispensable for thymic regulatory T cell production. *J Autoimmun* (2016) 75:141–9. doi: 10.1016/j.jaut.2016.08.002

34. Koble C, Kyewski B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J Exp Med* (2009) 206(7):1505–13. doi: 10.1084/jem.20082449
35. Martin-Gayo E, Sierra-Filardi E, Corbi AL, Toribio ML. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* (2010) 115(26):5366–75. doi: 10.1182/blood-2009-10-248260
36. Cedile O, Lobner M, Toft-Hansen H, Frank I, Wlodarczyk A, Irla M, et al. Thymic CCL2 influences induction of T-cell tolerance. *J Autoimmun* (2014) 55:73–85. doi: 10.1016/j.jaut.2014.07.004
37. Norment AM, Bogatzki LY, Gantner BN, Bevan MJ. Murine CCR9, a chemokine receptor for thymus-expressed chemokine that is up-regulated following pre-TCR signaling. *J Immunol* (2000) 164(2):639–48. doi: 10.4049/jimmunol.164.2.639
38. Baba T, Nakamoto Y, Mukaida N. Crucial contribution of thymic Sirp alpha+ conventional dendritic cells to central tolerance against blood-borne antigens in a CCR2-dependent manner. *J Immunol* (2009) 183(5):3053–63. doi: 10.4049/jimmunol.0900438
39. Cosway EJ, Ohigashi I, Schauble K, Parnell SM, Jenkinson WE, Luther S, et al. Formation of the Intrathymic Dendritic Cell Pool Requires CCL21-Mediated Recruitment of CCR7(+) Progenitors to the Thymus. *J Immunol* (2018) 201(2):516–23. doi: 10.4049/jimmunol.1800348
40. Lopes N, Charaix J, Cédile O, Sergé A, Irla M. Lymphotoxin α fine-tunes T cell clonal deletion by regulating thymic entry of antigen-presenting cells. *Nat Commun* (2018) 9(1):1262. doi: 10.1038/s41467-018-03619-9
41. Steimle A, Frick J-S. Molecular Mechanisms of Induction of Tolerant and Tolerogenic Intestinal Dendritic Cells in Mice. *J Immunol Res* (2016) 2016:1958650–. doi: 10.1155/2016/1958650
42. Toledano N, Gur-Wahnon D, Ben-Yehuda A, Rachmilewitz J. Novel CD47: SIRPalpha dependent mechanism for the activation of STAT3 in antigen-presenting cell. *PloS One* (2013) 8(9):e75595. doi: 10.1371/journal.pone.0075595
43. Cheng F, Wang HW, Cuenca A, Huang M, Ghansah T, Brayer J, et al. A critical role for Stat3 signaling in immune tolerance. *Immunity* (2003) 19(3):425–36. doi: 10.1016/s1074-7613(03)00232-2
44. Stransky LA, Forgac M. Amino Acid Availability Modulates Vacuolar H+-ATPase Assembly. *J Biol Chem* (2015) 290(45):27360–9. doi: 10.1074/jbc.M115.659128
45. Liberman R, Bond S, Shainheit MG, Stadecker MJ, Forgac M. Regulated assembly of vacuolar ATPase is increased during cluster disruption-induced maturation of dendritic cells through a phosphatidylinositol 3-kinase/mTOR-dependent pathway. *J Biol Chem* (2014) 289(3):1355–63. doi: 10.1074/jbc.M113.524561
46. Lee HC, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkappaB. *Proc Natl Acad Sci U S A* (2007) 104(3):914–9. doi: 10.1073/pnas.0607305104
47. Larghi P, Porta C, Riboldi E, Totaro MG, Carraro L, Orabona C, et al. The p50 subunit of NF-kappaB orchestrates dendritic cell lifespan and activation of adaptive immunity. *PloS One* (2012) 7(9):e45279. doi: 10.1371/journal.pone.0045279
48. Wurbel M-A, Le Bras S, Ibourk M, Pardo M, McIntire MG, Coco D, et al. CCL25/CCR9 Interactions Are Not Essential for Colitis Development but Are Required for Innate Immune Cell Protection from Chronic Experimental Murine Colitis. *Inflammatory Bowel Dis* (2014) 20(7):1165–76. doi: 10.1097/MIB.0000000000000059
49. Yamada A, Arakaki R, Saito M, Kudo Y, Ishimaru N. Dual Role of Fas/FasL-Mediated Signal in Peripheral Immune Tolerance. *Front Immunol* (2017) 8:403. doi: 10.3389/fimmu.2017.00403
50. Gandhi R, Farez MF, Wang Y, Kozoriz D, Quintana FJ, Weiner HL. Cutting edge: human latency-associated peptide+ T cells: a novel regulatory T cell subset. *J Immunol* (2010) 184(9):4620–4. doi: 10.4049/jimmunol.0903329
51. Ginhoux F, Guilliams M. Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity* (2016) 44(3):439–49. doi: 10.1016/j.immuni.2016.02.024
52. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* (2007) 204(8):1757–64. doi: 10.1084/jem.20070590
53. Rivollier A, He J, Kole A, Valatas V, Kelsall BL. Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J Exp Med* (2012) 209(1):139–55. doi: 10.1084/jem.20101387
54. Spadoni I, Iliev ID, Rossi G, Rescigno M. Dendritic cells produce TSLP that limits the differentiation of Th17 cells, fosters Treg development, and protects against colitis. *Mucosal Immunol* (2012) 5(2):184–93. doi: 10.1038/mi.2011.64
55. Strauch UG, Grunwald N, Obermeier F, Gurster S, Rath HC. Loss of CD103+ intestinal dendritic cells during colonic inflammation. *World J Gastroenterol* (2010) 16(1):21–9. doi: 10.3748/wjg.v16.i1.21
56. Lycke NY, Bemark M. The regulation of gut mucosal IgA B-cell responses: recent developments. *Mucosal Immunol* (2017) 10(6):1361–74. doi: 10.1038/mi.2017.62
57. Martinoli C, Chiavelli A, Rescigno M. Entry route of Salmonella typhimurium directs the type of induced immune response. *Immunity* (2007) 27(6):975–84. doi: 10.1016/j.immuni.2007.10.011
58. Cerutti A. The regulation of IgA class switching. *Nat Rev Immunol* (2008) 8(6):421–34. doi: 10.1038/nri2322
59. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Sci (N Y NY* (2006) 314(5802):1157–60. doi: 10.1126/science.1132742
60. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol* (2002) 3(9):822–9. doi: 10.1038/ni829
61. Conrad DH, Gibb DR, Sturgill J. Regulation of the IgE response. *F1000 Biol Rep* (2010) 2:14. doi: 10.3410/b2-14
62. Buela KA, Omenetti S, Pizarro TT. Cross-talk between type 3 innate lymphoid cells and the gut microbiota in inflammatory bowel disease. *Curr Opin Gastroenterol* (2015) 31(6):449–55. doi: 10.1097/mog.0000000000000217
63. Sonnenberg GF, Artis D. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. *Nat Med* (2015) 21(7):698–708. doi: 10.1038/nm.3892
64. Klose CSN, Flach M, Möhle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) 157(2):340–56. doi: 10.1016/j.cell.2014.03.030
65. Walker JA, Oliphant CJ, Englezakis A, Yu Y, Clare S, Rodewald H-R, et al. Bcl11b is essential for group 2 innate lymphoid cell development. *J Exp Med* (2015) 212(6):875–82. doi: 10.1084/jem.20142224
66. Hoyler T, Klose CSN, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity* (2012) 37(4):634–48. doi: 10.1016/j.immuni.2012.06.020
67. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J Exp Med* (2009) 206(1):35–41. doi: 10.1084/jem.20072713
68. Forkel M, van Tol S, Hoog C, Michaelsson J, Almer S, Mjosberg J. Distinct Alterations in the Composition of Mucosal Innate Lymphoid Cells in Newly Diagnosed and Established Crohn's Disease and Ulcerative Colitis. *J Crohns Colitis* (2019) 13(1):67–78. doi: 10.1093/ecco-jcc/jjy119
69. Lopez-Lastra S, Masse-Ranson G, Fiquet O, Darche S, Serafini N, Li Y, et al. A functional DC cross talk promotes human ILC homeostasis in humanized mice. *Blood Adv* (2017) 1(10):601–14. doi: 10.1182/bloodadvances.2017004358
70. Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, Hohl TM, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity* (2012) 36(2):276–87. doi: 10.1016/j.immuni.2011.12.011
71. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* (2008) 118(2):534–44. doi: 10.1172/jci33194
72. Tumanov AV, Koroleva EP, Guo X, Wang Y, Kruglov A, Nedospasov S, et al. Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. *Cell Host Microbe* (2011) 10(1):44–53. doi: 10.1016/j.chom.2011.06.002
73. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Sci (N Y NY)* (2014) 343(6178):1249288–. doi: 10.1126/science.1249288

74. Magri G, Cerutti A. Copycat innate lymphoid cells dampen gut inflammation. *Cell Res* (2015) 25(9):991–2. doi: 10.1038/cr.2015.79
75. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific $CD4^+$ T cells. *Sci (N Y NY)* (2015) 348(6238):1031–5. doi: 10.1126/science.aaa4812
76. Kulkarni N, Meitei HT, Sonar SA, Sharma PK, Mujeeb VR, Srivastava S, et al. CCR6 signaling inhibits suppressor function of induced-Treg during gut inflammation. *J Autoimmun* (2018) 88:121–30. doi: 10.1016/j.jaut.2017.10.013

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 26 October 2020
doi: 10.3389/fimmu.2020.576752

Interferon-γ/Interleukin-27 Axis Induces Programmed Death Ligand 1 Expression in Monocyte-Derived Dendritic Cells and Restores Immune Tolerance in Central Nervous System Autoimmunity

*Giacomo Casella[1], Javad Rasouli[1], Rodolfo Thome[1], Hélène C. Descamps[2], Asrita Vattikonda[1], Larissa Ishikawa[1], Alexandra Boehm[1], Daniel Hwang[1], Weifeng Zhang[1], Dan Xiao[1], Jeongho Park[3,4], Guang-Xian Zhang[1], Jorge I. Alvarez[3], Abdolmohamad Rostami[1] and Bogoljub Ciric[1]**

[1] Department of Neurology, Thomas Jefferson University, Philadelphia, PA, United States, [2] Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [3] Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, United States, [4] College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, Chuncheon, South Korea

OPEN ACCESS

Edited by:
Dipyaman Ganguly,
Indian Institute of Chemical Biology, India

Reviewed by:
Sin-Hyeog Im,
Pohang University of Science and Technology, South Korea
Sylvaine You,
Institut National de la Santé et de la Recherche Médicale, France

***Correspondence:**
Bogoljub Ciric
bogoljub.ciric@jefferson.edu

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: 26 June 2020
Accepted: 02 September 2020
Published: 26 October 2020

Citation:
Casella G, Rasouli J, Thome R, Descamps HC, Vattikonda A, Ishikawa L, Boehm A, Hwang D, Zhang W, Xiao D, Park J, Zhang G-X, Alvarez JI, Rostami A and Ciric B (2020) Interferon-γ/Interleukin-27 Axis Induces Programmed Death Ligand 1 Expression in Monocyte-Derived Dendritic Cells and Restores Immune Tolerance in Central Nervous System Autoimmunity. Front. Immunol. 11:576752. doi: 10.3389/fimmu.2020.576752

Antigen (Ag)-specific tolerance induction by intravenous (i. v.) injection of high-dose auto-Ags has been explored for therapy of autoimmune diseases, including multiple sclerosis (MS). It is thought that the advantage of such Ag-specific therapy over non-specific immunomodulatory treatments would be selective suppression of a pathogenic immune response without impairing systemic immunity, thus avoiding adverse effects of immunosuppression. Auto-Ag i.v. tolerance induction has been extensively studied in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, and limited clinical trials demonstrated that it is safe and beneficial to a subset of MS patients. Nonetheless, the mechanisms of i.v. tolerance induction are incompletely understood, hampering the development of better approaches and their clinical application. Here, we describe a pathway whereby auto-Ag i.v. injected into mice with ongoing clinical EAE induces interferon-gamma (IFN-γ) secretion by auto-Ag-specific $CD4^+$ T cells, triggering interleukin (IL)-27 production by conventional dendritic cells type 1 (cDC1). IL-27 then, *via* signal transducer and activator of transcription 3 activation, induces programmed death ligand 1 (PD-L1) expression by monocyte-derived dendritic cells (moDCs) in the central nervous system of mice with EAE. PD-L1 interaction with programmed cell death protein 1 on pathogenic $CD4^+$ T cells leads to their apoptosis/anergy, resulting in disease amelioration. These findings identify a key role of the IFN-γ/IL-27/PD-L1 axis, involving T cells/cDC1/moDCs in the induction of i.v. tolerance.

Keywords: peripheral tolerance, monocytes, experimental autoimmune encephalitis, PD-L1, cytokines

INTRODUCTION

Autoimmune diseases develop due to a break in immune tolerance toward certain auto-antigens (auto-Ags). It follows that the healthy state could be achieved by restoring peripheral immune tolerance toward those auto-Ags. Devising therapies based on the restoration of Ag-specific immune tolerance induction has been a long-standing goal for treatment of autoimmune diseases, including multiple sclerosis (MS) (1). One such approach relies on intravenous (i.v.) injection of free myelin-derived auto-Ags that are targets of autoimmune response. This approach, and variants thereof, have been proven beneficial in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (1). Some clinical trials have confirmed that repeatedly i.v. injecting large doses of myelin auto-Ag can be safe and beneficial to a subset of MS patients (1). In comparison with non-specific immunomodulatory therapies currently in use, the principal advantage of Ag-specific therapy would be that it suppresses harmful autoimmune response while sparing the rest of the immune system. This would eliminate side effects and adverse events due to systemic immunosuppression caused by non-specific immunomodulation.

Even though certain key players in i.v. tolerance induction, such as interleukin (IL)-10, IL-27, and programmed death ligand 1 (PD-L1), have been identified (2, 3), its complete mechanisms have not been elucidated. This lack of specific knowledge also includes the cell types involved, sequence of their interactions, and relative relevance of the periphery vs. the central nervous system (CNS) in tolerance induction and maintenance over time. A more thorough understanding of these mechanisms will be helpful in developing better Ag-specific therapies for MS and possibly other autoimmune diseases.

Consistent with the suppressive role of IL-10 in EAE development (4), i.v. tolerance induction in EAE requires IL-10. Tolerization by i.v. injection of an auto-Ag elicits IL-10 production, and blockade of IL-10 signaling precludes tolerance induction (2, 5, 6).

The lack of IL-27 signaling leads to the development of more severe EAE (7), and treatment with recombinant IL-27 suppresses EAE (8–10), demonstrating its anti-inflammatory role in EAE. The anti-inflammatory effects of IL-27 encompass inhibition of Th17 cell development; suppression of granulocyte-macrophage colony-stimulating factor (GM-CSF) expression; induction of PD-L1, CD39, and IL-10 expression; and enhancement of Treg development and function (8–10). We have shown that IL-27 is necessary for induction of i.v. tolerance in EAE (3); in particular, IL-27 signaling in DCs was required for tolerance induction, whereas its signaling in T cells was not. IL-27-dependent tolerance induction relied on cooperation of distinct subsets of spleen DCs with the ability to induce T cell-derived IL-10 and interferon-gamma (IFN-γ) (3).

Programmed cell death protein 1 (PD-1) and its ligands, PD-L1 and PD-L2, regulate the balance between T cell activation and immune tolerance (11, 12). The majority of $CD4^+$ T cells in the CNS of mice with EAE express PD-1, while PD-L1 and PD-L2 are differentially expressed by populations of Ag-presenting cells (APC) (13). PD-$1^{-/-}$ and PD-$L1^{-/-}$ mice develop atypically severe EAE, with enhanced T cell proliferation and increased production of inflammatory cytokines (14, 15). Genetic deficiency in PD-L2 did not lead to more severe EAE (14), suggesting that PD-L1 is a dominant inhibitory PD-1 ligand in EAE development. In contrast, blockade of PD-L2, but not PD-L1, in advanced EAE in C57BL/6 mice led to worsening of disease, indicating that these two PD-1 ligands, or possibly cell types that express them, have distinct roles in regulating different stages of EAE (2, 16, 17). In regard to i.v. tolerance induction in EAE, it has been shown that tolerization induces PD-L1 expression by APCs and that PD-1 blockade abrogates tolerance induction (2, 18).

IFN-γ is a cytokine released by almost all activated immune cells, with natural killer (NK) and T cells being its major sources (19). Although IFN-γ has been traditionally considered a pro-inflammatory cytokine, it is now clear that IFN-γ also has prominent anti-inflammatory roles that balance its possibly damaging inflammatory effects (20). Numerous studies have firmly established that IFN-γ suppresses EAE; mice lacking IFN-γ signaling develop severe EAE, and mouse strains resistant to EAE become susceptible (21–23). Consistent with this, IFN-γ production by myelin-specific $CD4^+$ T cells is not required for their encephalitogenicity (24), IFN-γ-deficient $CD4^+$ T cells could be notably more pathogenic than their IFN-γ-sufficient counterparts (25), and IFN-γ production by encephalitogenic T cells in the CNS is required for recovery from EAE (26). Further, an increase in IFN-γ levels in the CNS of mice with EAE leads to disease suppression (27, 28). Taken together, these findings indicate that IFN-γ could be important in EAE suppression by i.v. tolerance induction as well, a possibility that has not been explored.

Here we show that i.v. administration of auto-Ag (free encephalitogenic peptide) halts EAE progression by inducing PD-L1 expression in CNS monocyte-derived dendritic cells (moDCs) *via* an IFN-γ/IL-27-dependent mechanism. Blockade of PD-L1, but not PD-L2, or the lack of PD-1 in $CD4^+$ T cells precluded i.v. tolerance induction. The lack of IFN-γ in $CD4^+$ T cells, or IFN-γR in conventional DCs type 1 (cDC1), abrogated IL-27 production and PD-L1 expression by moDCs. Collectively, our data reveal a mechanism of Ag-dependent induction of PD-L1 expression in moDCs that in turn suppresses Ag-specific Th cell responses and ameliorates CNS autoimmunity.

MATERIALS AND METHODS

Mice

C57BL/6, B6.Ly5.1 ($CD45.1^+$), $RAG1^{-/-}$, PD-$1^{-/-}$, 2D2, Zbtb46-iDTR, IFN-$\gamma^{-/-}$, IFN-γR$\alpha^{-/-}$, GREAT (IFN-γ reporter), *Ccr2*$^{-/-}$, *Wsx*$^{-/-}$, and *Stat3*^{mut} mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). IL-27p28 reporter mice were a gift of Dr. Ross M. Kedl (University of Colorado). Mice were kept in specific pathogen-free conditions with a maximum of 5 mice per cage, in 12/12 h of light/dark cycles and food *ad libitum* throughout the experimental procedures. Every effort was made to minimize suffering of mice. Experimental protocols using mice were approved by

the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Generation of BMDCs

STAT1$^{-/-}$, STAT3$^{-/-}$, and WT BMDCs were generated according to a previously described protocol (3). Briefly, BM cells were seeded at 2×10^6 cells/mL in Petri dish in complete IMDM supplemented with 100 ng/mL of recombinant mouse Flt-3 (R&D Systems, Minneapolis, MN, USA). Culture medium was changed every 3 days. Maturation of the DCs was induced with LPS (100 ng/mL) for 16 h. At day 9 after starting the culture, DCs were enriched by anti-Flt-3-biotin Ab and anti-biotin microbeads (Miltenyi Biotec, CA, USA), and CD11c$^+$MHCII$^+$ cells were then FACS-sorted.

IFN-γRα$^{-/-}$ and WT cDCs were generated from BM cells following a protocol described in (29) with slight modifications.

Ag-presentation Assays

Naive CD4$^+$ T cells from spleens of 2D2 mice were isolated using magnetic beads (Naive CD4$^+$ T cell isolation kit, Miltenyi Biotec, CA, USA). 2×10^5 naive CD4$^+$ T cells were added to each well of the cell culture plate containing moDCs, cDC1, or BMDCs (ratio of 1 DC: 10 T cells) and plates were incubated at 37°C in the presence of MOG_{35-55} peptide (20 μg/mL) and anti-PD-L1 MAb (1 μg/mL; clone 10F.9G2, BioXCell). Cells were collected after 72 h and analyzed by flow cytometry, while cytokine concentrations in culture supernatants were measured by ELISA.

EAE and i.v. Tolerance Induction

Anesthetized mice were subcutaneously injected with 200 μL of an emulsion containing 200 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK, Genscript, NJ, USA) in PBS and equal volume of Complete Freund's adjuvant supplemented with 10 mg/mL of heat-killed *Mycobacterium tuberculosis* H37Ra. Additionally, mice were intraperitoneal (i.p.) injected with 200 ng of pertussis toxin at immunization time and 48 h later. Mice were weighed and scored for clinical signs daily. Clinical assessment of EAE was performed according to the following scoring criteria: 0, healthy; 1, limp tail; 2, ataxia and/or paresis of hindlimbs; 3, paralysis of hindlimbs and/or paresis of forelimbs; 4, tetraparalysis; and 5, moribund or death (30).

i.v. tolerance was induced in mice after onset of clinical disease by injections of 200 μg MOG_{35-55} in PBS every third day, 3 times in total. Control mice received PBS only (3).

Bone Marrow Chimeras

B6.Ly5.1 (CD45.1$^+$) congenic mice were lethally irradiated with 2×2.5 Gy with an 8 h interval between irradiation and were then i.v. injected with 5×10^6 CD45.2$^+$ BM cells from WT, or Zbtb46-DTR donors. Recipient mice were in other experiments reconstituted with 1:1 mixture (total 1×10^7 cells) of BM cells from *Wsx*$^{-/-}$ and *Ccr2*$^{-/-}$ mice, or with mixture of BM cells from *Stat3*^{mut} and *Ccr2*$^{-/-}$ mice. Mice were allowed to reconstitute for 6–8 weeks prior to use.

DT Ablation

Diphtheria toxin (DTX; Sigma-Aldrich) was administered i.p. at 1 μg/20 g of mouse weight in 200 μl of PBS, 1 day before i.v. injection of MOG_{35-55}. Mice received three injections of DTX once every 3 days.

PD-L1, PD-L2, and IFN-γ Blockade

Mice with EAE were i.p. injected with 200 μg/mouse of αPD-L1 MAb (clone 10F.9G2, BioXCell), or with 200 μg/mouse of αPD-L2 MAb (clone TY25, BioXCell), or with 150 μg/mouse of αIFN-γ MAb (clone R4-6A2, BioXCell) 1 day before i.v. injection of MOG_{35-55}. Mice received two MAb injections, 3 days apart in each treatment.

Ag-Specific Recall Response

Spleens of mice with EAE were dissociated through a 70 μm strainer to prepare single-cell suspensions in complete IMDM, containing 10% heat-inactivated fetal bovine serum, penicillin (100 U), streptomycin (10 μg/mL), L-glutamine (0.3 mg/mL), and 2-mercaptoethanol (55 μM). After treatment with RBC lysis buffer (Biolegend, CA, USA), cells were extensively washed with complete IMDM by centrifugation at 1,300 rpm for 5 min at 4°C and the cell density was adjusted to 2×10^6 /mL. One hundred microliter of adjusted cell suspension was added to each well of a 96-well plate. MOG_{35-55} was added to a final concentration of 20 μg/mL. Cells were incubated at 37°C for 3 days. As negative control, cells were cultured without MOG_{35-55}. Cell culture supernatants were collected and stored at −20°C until use, and cells were analyzed for proliferation and cytokine production by flow cytometry.

Reconstitution of WT and RAG1$^{-/-}$ Mice

WT mice with EAE received i.v. 2×10^6 FACS-sorted CD11b$^+$ CD11c$^+$ Ly6c^{high} MHCII$^+$ cells from the CNS of WT mice with EAE previously i.v. injected with MOG_{35-55} or PBS. CD45.1$^+$ mice reconstituted with CD45.2$^+$ BM cells from WT, or Zbtb46-iDTR donors, received i.v. 2×10^6 of *in vitro* Flt-3-differentiated cDCs. RAG1$^{-/-}$ mice were i.v. reconstituted with 5×10^6 magnetic bead-isolated total CD4$^+$ T cells from spleens of WT, PD1$^{-/-}$, or IFN-γ$^{-/-}$ mice. After 72 h of adoptive transfer, mice were immunized for EAE induction.

Isolation of CNS Infiltrating Leukocytes

Brain and spinal cord tissues were incubated for 30 min at 37°C with 0.4 mg/mL type IV collagenase (Sigma-Aldrich) and dissociated by passing through a 19-gouge needle. Cells were enriched by centrifugation on a Percoll gradient as previously described (31).

Flow Cytometry and Cell Sorting

Flow cytometry was performed using a FACSaria II (Becton Dickinson) and analyzed with FlowJo software (Tree Star). Fluorochrome-conjugated MAbs specific for: CD45 (clone 30-F11), CD45.1 (A20), CD11b (M1/70), CD3 (17A2), CD8α (53-6.7) CD4 (RM4-5), CD19 (1D3/CD19), CD11c (N418), CD26 (H194-112), CD88 (20/70), CD172α (P84), PDCA1 (927), Ly6c (AL-21), F4/80 (MB8), Ly6g (1A8), MHC-II (M5/114.15.2), PD-1 (29F.1A12), PD-L1 (10F.9G2), PD-L2 (TY25), Caspase 3, and

Annexin V were purchased either from BD Biosciences, R&D, Biolegend, Santa Cruz, or Abcam.

For intracellular staining, cells were stimulated for 4 h with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml, Sigma-Aldrich) and ionomycin (500 ng/ml, Sigma-Aldrich) in the presence of GolgiPlug (1:1,000, BD Pharmigen), permeabilized using a Cytofix/Cytoperm Plus kit (BD Bioscience) and stained with the following fluochrome-conjugated MAbs: GM-CSF (MP1-22E9), IL-17A (TC11-18H10.1), and IFN-γ (XMG1.2) from Biolegend and BD Pharmingen. Dead cells were excluded using L/D stain (BD Pharmingen). Data were acquired on a FACSAria Fusion (BD Biosciences) and analyzed using FlowJo software (TreeStar).

ELISA

Supernatants from cell cultures were kept at −20°C until use. Cytokine concentrations in culture supernatants were measured with sandwich enzyme-linked immunosorbent assay (ELISA) using commercial kits, following the manufacturer's recommendation (R&D Systems, Minneapolis, MN, USA).

qPCR

Total RNA was extracted from moDCs, $CD4^+$ T cells, and cDC1 with RNeasy Mini Kit (Qiagen), whereas from total CNS and spleen with Trizol (Invitrogen). Genomic DNA was removed by treatment with DNAse I type (Qiagen). cDNA synthesis was performed using ThermoscriptTM RT-PCR system (Invitrogen). Apoptosis (cat# 4413255), Jak/Stat signaling (cat# 4391524) arrays, *Il27ra*, (Mm00497259_m1), and *Gapdh* (4352339E). mRNA levels were measured by real-time RT-PCR (Applied Biosystems, Invitrogen). The 2–ΔΔCT method was used to calculate relative changes in gene expression (32).

Statistical Analysis

Statistical analysis was performed by GraphPad Prism 8 software. Statistical evaluations are expressed as mean ± s.d. or mean ± s.e.m., as appropriate. Results were analyzed using Two- or One-way ANOVA and posttested with Bonferroni, and with unpaired, two-tailed Student's *t*-test. Statistical significance was ranked $^*p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$; $^{****}p < 0.00001$.

RESULTS

Intravenous Tolerance Induction in Experimental Autoimmune Encephalomyelitis Is Dependent on Programmed Death Ligand 1 and Programmed Cell Death Protein 1, but Not on Programmed Death Ligand 2

We and others have reported that i.v. delivery of auto-Ag in mice with EAE induces expression of PD-L1 by APCs (2, 3). Given the importance of PD-1 and its ligands in immune tolerance, we investigated their role in i.v. tolerance induction in EAE. Mice were immunized with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ for EAE induction and i.v. injected with 200 μg of MOG_{35-55} in phosphate-buffered saline (PBS), or with PBS, at the onset of clinical disease; two more doses of MOG_{35-55} and PBS were injected 3 days apart (**Supplementary Figures 1A,B**). Mice were sacrificed at 21 days post immunization (d.p.i.), and cells isolated from their CNS were analyzed by flow cytometry. t-distributed stochastic neighbor embedding (t-SNE) analysis (33) identified eight populations of $CD45^+$ $MHCII^+$ cells: cDC1, cDC2, microglia, plasmacytoid DCs (pDCs), moDCs, macrophages (MΦ), neutrophils, and B cells (**Figure 1A**). MOG_{35-55}-treated mice had increased numbers of PD-$L1^+$ and PD-$L2^+$ cells compared with PBS-treated mice. Next, we investigated which of these cells expressed PD-L1 and PD-L2. moDCs (75%) were the bulk of PD-$L1^+$ cells, whereas in control mice, cDC1 (58%) and cDC2 (33%) were the most abundant PD-$L1^+$ cells (**Figure 1B**). In mice that received MOG_{35-55}, PD-L2 was mostly expressed by neutrophils (50%) and MΦ (35%), whereas in control mice, PD-L2 was expressed by microglia (64%) and cDC2 (34%) (**Figure 1C**). These data show that i.v. delivery of auto-Ag induces a robust PD-L1 expression by moDCs and PD-L2 expression by neutrophils and MΦ. We also found increased numbers of PD-$L1^+$ and PD-$L2^+$ cells among lymph node and splenic cells of MOG_{35-55}-tolerized mice (data not shown).

We then tested the role of PD-L1 and PD-L2 in tolerance induction by i.p. injecting anti-PD-L1 or anti-PD-L2 MAbs 24 h before i.v. injecting MOG_{35-55}. Treatment with anti-PD-L1 MAb precluded tolerance induction (**Figures 1D,E**) and led to an increase in the number of leukocytes in the CNS (**Figure 1F**), whereas treatment with anti-PD-L2 MAb did not have an effect (**Figures 1G–I**). Further, we transplanted PD-$1^{-/-}$ or wild-type (WT) $CD4^+$ T cells into $RAG1^{-/-}$ mice and induced EAE in them. MOG_{35-55}/i.v. treatment suppressed EAE in mice with WT $CD4^+$ T cells, but not in mice with PD-$1^{-/-}$ $CD4^+$ T cells (**Figures 1J–L**). These data demonstrate that PD-L1 and PD-1 are critical for i.v. tolerance induction in ongoing EAE, whereas PD-L2 does not play a role.

Monocyte-Derived Dendritic Cells From the Central Nervous System of Tolerized Mice With Experimental Autoimmune Encephalomyelitis Are Suppressive via Programmed Death Ligand 1

Given that tolerized mice had increased numbers of apoptotic annexin V^+ $CD4^+$ T cells (**Supplementary Figures 1C,D**) and PD-$L1^+$ moDCs in the CNS (**Figure 1B**), we investigated a correlation between their numbers. There was a robust positive correlation of apoptotic $CD4^+$ T cells with PD-$L1^+$ moDCs (**Supplementary Figure 1E**), but not with PD-$L2^+$ moDCs (**Supplementary Figure 1F**) or PD-$L2^+$ neutrophils (data not shown). This suggests that PD-$L1^+$ moDCs mediate apoptosis of T cells.

We next evaluated the effect of moDCs on myelin-specific $CD4^+$ T cells *ex vivo*. $MHCII^+$ $Ly6c^{high}$ moDCs were sorted from the CNS of mice with EAE treated with MOG_{35-55}/i.v. and co-cultured with naive $CD4^+$ T cells expressing a transgenic T

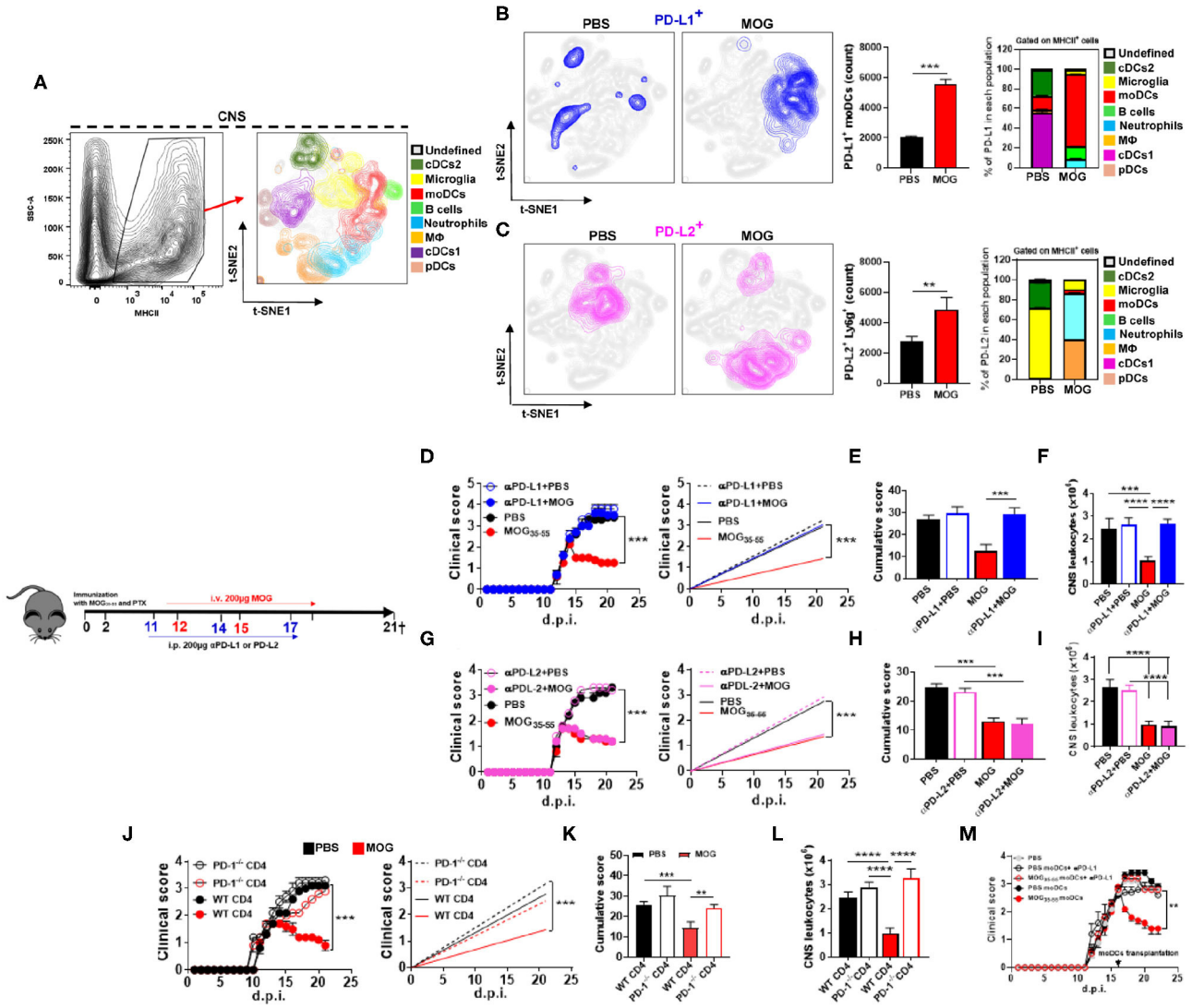


FIGURE 1 | Intravenous administration of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ induces programmed death ligand 1 (PD-L1) expression in central nervous system (CNS) monocyte-derived dendritic cells (moDCs) and suppresses experimental autoimmune encephalomyelitis (EAE) in a programmed cell death protein 1 (PD-1)/PD-L1 manner. C57BL/6 wild-type (WT) mice ($n = 10$/group each experiment) were immunized with MOG_{35-55} for EAE induction and starting from disease onset i.v. injected with 200 μg of MOG_{35-55} in PBS every 3 days. Mice were sacrificed 21 days post immunization (d.p.i.) and $CD45^+$ $MHCII^+$ cells from the CNS were analyzed by flow cytometry. **(A)** Eight populations were identified: microglia ($CD11b^+$ $CD45^{low}$), monocytes ($CD11b^+$ $CD11c^+$ $Ly6c^{high}$ $Ly6g^-$), macrophages ($CD11b^+$ $Ly6c^{low}$ $Ly6g^-$ $F4/80^{high}$), cDC1 ($CD11b^+$ $CD11c^+$ $Ly6c^{med}$ $CD26^+$), cDC2 ($CD11b^+$ $CD11c^+$ $Ly6c^{med}$ $CD172\alpha^+$), plasmacytoid DCs (pDCs; Lin^- $PDCA1^{high}$), and B cells ($CD19^+$). **(B,C)** t-SNE graphs showing the expression and percentage of PD-L1$^+$ **(B)** and PD-L2 **(C)** cells from the CNS of PBS- or MOG_{35-55}-treated EAE mice. **(D)** Mice with EAE ($n = 5$–7/group in each experiment) were i.p. injected with blocking anti-PD-L1 MAb (200 μg/mouse/injection; clone 10F.9G2), or isotype control MAb, on 11, 14, and 17 d.p.i. MOG_{35-55} or PBS was i.v. injected on 12, 15, and 18 d.p.i. **(E)** Cumulative disease score for mice described in **(D)**. **(F)** Mice described in **(D)** were sacrificed at day 21 p.i. and the numbers of $CD45^+$ leukocytes obtained from their CNS were determined by flow cytometry. **(G)** Mice with EAE ($n =$ 5–7/group in each experiment) were i.p. injected with blocking anti-PD-L2 MAb (200 μg/mouse/injection; clone TY25), or isotype control MAb, as described in **(D)**. **(H)** Cumulative disease score for mice described in **(G)**. **(I)** Mice described in **(G)** were sacrificed at day 21 p.i. and the numbers of $CD45^+$ leukocytes obtained from their CNS were determined by flow cytometry. **(J)** $RAG1^{-/-}$ mice were reconstituted with 5×10^6 total $CD4^+$ T cells from WT or PD-$1^{-/-}$ mice ($n = 10$/group in each experiment); 72 h post reconstitution, recipient mice were immunized for EAE induction and MOG_{35-55} or PBS was injected i.v. three times, starting from EAE onset. **(K)** Cumulative disease score for mice described in **(J)**. **(L)** Mice described in **(J)** were sacrificed at day 21 p.i. and the numbers of $CD45^+$ leukocytes obtained from their CNS were determined by flow cytometry. **(M)** Recipient WT mice with EAE were transplanted at the peak of disease with 2×10^6 moDCs from the CNS of donor mice with EAE that were previously i.v. tolerized with MOG_{35-55} or PBS ($n = 5$/group in each experiment). Groups of recipient mice with EAE received moDCs that were pretreated for 1 h with anti-PD-L1 MAb (1 μg/ml). All data are representative of at least two experiments and symbols depict mean ± SEM. Analyses between two groups were carried out by Student's *t*-test and between four groups by one-way ANOVA with Bonferroni post-test **(E,F,H,I,K,L)**. EAE experiments were analyzed by two-way ANOVA with Bonferroni's multiple comparison. Values of $^{**}P < 0.001$, $^{***}P < 0.0001$, and $^{****}P < 0.00001$ were considered significant.

cell receptor for MOG_{35-55} from 2D2 mice. Anti-PD-L1 MAb was added in some co-cultures. In comparison with moDCs from control mice, moDCs from MOG_{35-55}-tolerized mice induced lower T cell proliferation and lower GM-CSF and IL-17A production, but greater IFN-γ and PD-1 expression and annexin V staining by T cells (**Supplementary Figures 1G–I**). We also measured a larger quantity of regulatory cytokines, IL-27 and IL-10, in supernatants of these co-cultures compared with controls (**Supplementary Figure 1J**). In the presence of anti-PD-L1 MAb, moDCs did not reduce T cell proliferation or induce their apoptosis, demonstrating that PD-L1 expressed by moDCs limits myelin-specific $CD4^+$ T cell responses *in vitro*.

To validate the immunosuppressive phenotype of MOG_{35-55}/i.v.-induced moDCs *in vivo*, we transplanted moDCs from the CNS of tolerized mice with EAE into mice with ongoing EAE. Control mice received either moDCs from PBS/i.v. mice, PBS, or moDCs from MOG_{35-55}/i.v. mice that were pretreated for 1 h with anti-PD-L1 MAb. The transfer of moDCs from MOG_{35-55}-treated donors, but not from those that were PBS-treated, led to recovery from the disease, and anti-PD-L1 MAb pretreatment of moDCs from MOG_{35-55}/i.v. donors precluded their suppressive effect on EAE in recipient mice (**Figure 1M**). These data show that moDCs of MOG_{35-55}-treated mice with EAE are suppressive *via* PD-L1 *in vitro* and *in vivo*.

Interferon-γ Secreted by $CD4^+$ T Cells Is Necessary for Experimental Autoimmune Encephalomyelitis Suppression Upon Myelin Oligodendrocyte $Glycoprotein_{35-55}$/Intravenous Treatment

Given that IFN-γ suppresses EAE (28, 34) and induces PD-L1 expression (35), we tested whether IFN-γ plays a role in tolerance induction. We injected anti-IFN-γ MAb into mice after EAE onset, 24 h before MOG_{35-55}/i.v. injection (**Figure 2A**). Anti-IFN-γ-treated mice developed a severe disease that did not respond to MOG_{35-55}/i.v. treatment (**Figures 2B,C**). These mice had markedly increased numbers of leukocytes in the CNS compared with control MOG_{35-55}/i.v.-treated mice (**Figure 2D**). Anti-IFN-γ-treated mice also had lower expression of $PD\text{-}L1^+$ in moDCs, reduced numbers of apoptotic $CD4^+$ T cells, and greater frequencies of $GM\text{-}CSF^+$ $IL\text{-}17A^+$ $CD4^+$ T cells (**Figures 2E–I**). Consistent with this observation, we found higher concentrations of GM-CSF and IL-17A in culture supernatants from MOG_{35-55}-stimulated splenocytes of the above anti-IFN-γ-treated mice compared with controls (**Figures 2J,K**); however, while MOG_{35-55}/i.v. treatment resulted in an increase of IL-10, and especially IL-27 production, treatment with anti-IFN-γ MAb precluded these increases (**Figures 2L,M**). We next determined the kinetics of IFN-γ expression upon MOG_{35-55} injection into mice with EAE. *Ifng* mRNA levels, both in the CNS and spleen, were highest at 6 h after the injection and declined to base levels by 12 h post injection (**Figures 3A,B**). These data show that IFN-γ plays a critical role in the induction of i.v. tolerance in EAE.

To identify the cellular sources of MOG_{35-55}/i.v.-induced IFN-γ, we induced EAE in IFN-γ reporter mice (express GFP from the IFN-γ gene), injected them with MOG_{35-55}/i.v. at disease onset, and 3 h after the injection analyzed their CNS. IFN-γ was primarily produced by $CD4^+$ T cells, whereas in control mice injected with PBS, both cDCs and $CD4^+$ T cells produced IFN-γ (**Figure 3C**). We then investigated whether $CD4^+$ T cells are a relevant source of IFN-γ in tolerance induction. To this end, we reconstituted $RAG1^{-/-}$ mice with WT or $IFN\text{-}\gamma^{-/-}$ $CD4^+$ T cells and immunized them for EAE induction; mice received PBS or MOG_{35-55}/i.v. at disease onset. MOG_{35-55}/i.v. significantly suppressed disease in mice with WT $CD4^+$ T cells, whereas it exacerbated the disease in mice with $IFN\text{-}\gamma^{-/-}$ T cells (70% of mice died) (**Figures 3D,E**). Consistent with clinical outcome, mice with $IFN\text{-}\gamma^{-/-}$ T cells had reduced $PD\text{-}L1^+$ expression in CNS moDCs compared with mice with WT T cells (**Figure 3H**). Moreover, splenocytes of mice with $IFN\text{-}\gamma^{-/-}$ T cells upon *in vitro* activation produced significantly less IL-27 compared with control mice (**Figure 3I**). These data show that IFN-γ secretion by $CD4^+$ T cells is required for EAE suppression by MOG_{35-55}/i.v. treatment.

The Lack of Interferon-γ Signaling in Conventional Dendritic Cells Type 1 Precludes Their Interleukin-27 Expression in Intravenous Tolerance Induction in Experimental Autoimmune Encephalomyelitis

Blocking IFN-γ resulted in reduced IL-27 production, prompting us to search for the cellular source of IL-27 upon MOG_{35-55}/i.v. treatment. We induced EAE in IL-27 reporter mice (express GFP from the IL-27p28 gene) and injected them with MOG_{35-55} at disease onset. IL-27 production in the spleen and CNS peaked at 16 h after the injection (**Figures 4A,B**), and among cells from the CNS, the primary IL-27-producing cells were cDC1 (∼80% of GFP^+ cells), while in PBS-treated mice, moDCs also produced IL-27 (**Figure 4C**).

We next investigated whether the lack of IFN-γ signaling in cDC1 affects IL-27 production and compromises tolerance induction. We generated a Zbtb46-DTR ($CD45.2^+$)→ $CD45.1^+$ bone marrow (BM) chimera mice in which cDC1 can be depleted with diphtheria toxin (DTX) (36) (**Supplementary Figure 2A**). WT ($CD45.2^+$)→ $CD45.1^+$ BM chimeras served as control. We immunized chimera mice for EAE induction and depleted cDC1 by administering DTX, starting at disease onset and then every other day. We then transplanted *in vitro* Flt3-differentiated WT or $IFN\text{-}\gamma R^{-/-}$ cDC1 into these DTX-treated chimera mice with EAE (**Figure 4D**). Mice were treated with DTX and transplanted with cDC1 twice. Moreover, 24 h post cDC1 transplantation, mice were injected with PBS or MOG_{35-55}, three times, 3 days apart. MOG_{35-55}/i.v. treatment had no effect in mice that received $IFN\text{-}\gamma R^{-/-}$ cDC1, whereas in mice with WT cDC1, it significantly suppressed disease (**Figure 4D**). We also found a reduced

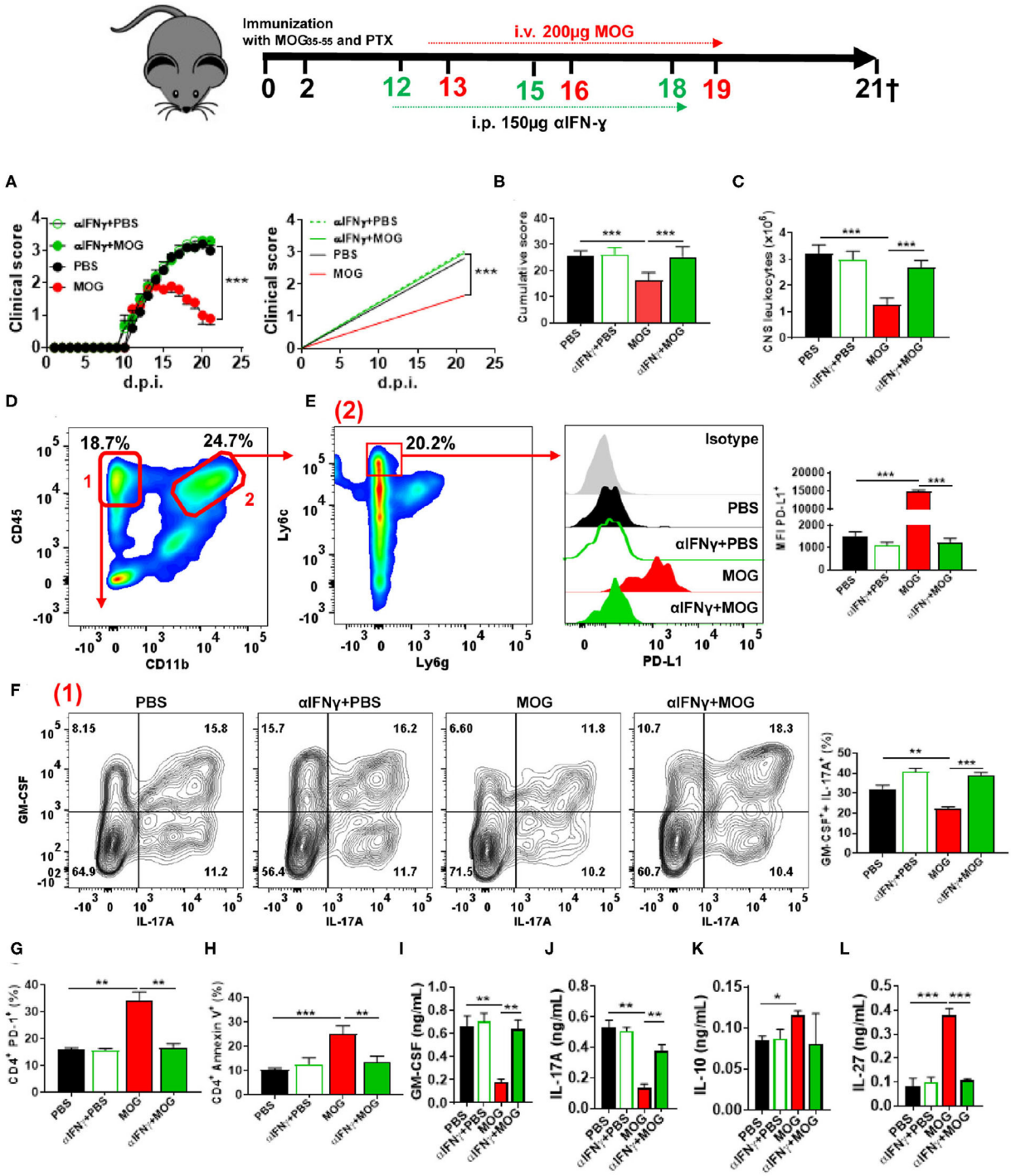


FIGURE 2 | IFN-γ is critical for i.v. tolerance induction in EAE. **(A)** Mice with EAE (n = 5–7/group in each experiment) were i.p. injected with blocking anti-IFN-γ MAb (150 µg/mouse/injection; clone H22), or isotype control MAb, on 12, 15, and 18 d.p.i. MOG_{35-55} or PBS was i.v. given on 13, 16, and 19 d.p.i. **(B)** Cumulative disease score for mice described in **(A)**. **(C)** Mice described in **(A)** were sacrificed on day 21 p.i. and the numbers of $CD45^+$ leukocytes in their CNS were determined by flow cytometry. **(D)** Flow cytometry plot showing lymphoid (1) and infiltrating myeloid (2) cells from the CNS of mice shown in **(A)**. **(E)** MFI of $PD\text{-}L1^+$ moDCs from the CNS of mice described in **(A)**. Frequencies of $GM\text{-}CSF^+$ $IL\text{-}17A^+$ **(F)**, $PD\text{-}1^+$ **(G)**, and annexin V^+ **(H)** $CD4^+$ T cells from the CNS of mice described in **(A)**. **(J–M)** Cytokine concentrations in supernatants from cultures of spleen cells from mice described in **(A)** stimulated for 72 h with MOG_{35-55}. Cytokine concentrations were measured by ELISA. All data are representative of at least two experiments, and symbols depict mean ± SEM. Analysis between four groups was carried out by one-way ANOVA with Bonferroni post-test. EAE experiments in **(A)** were analyzed by two-way ANOVA with Bonferroni's multiple comparison. Values of $^*P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ were considered significant.

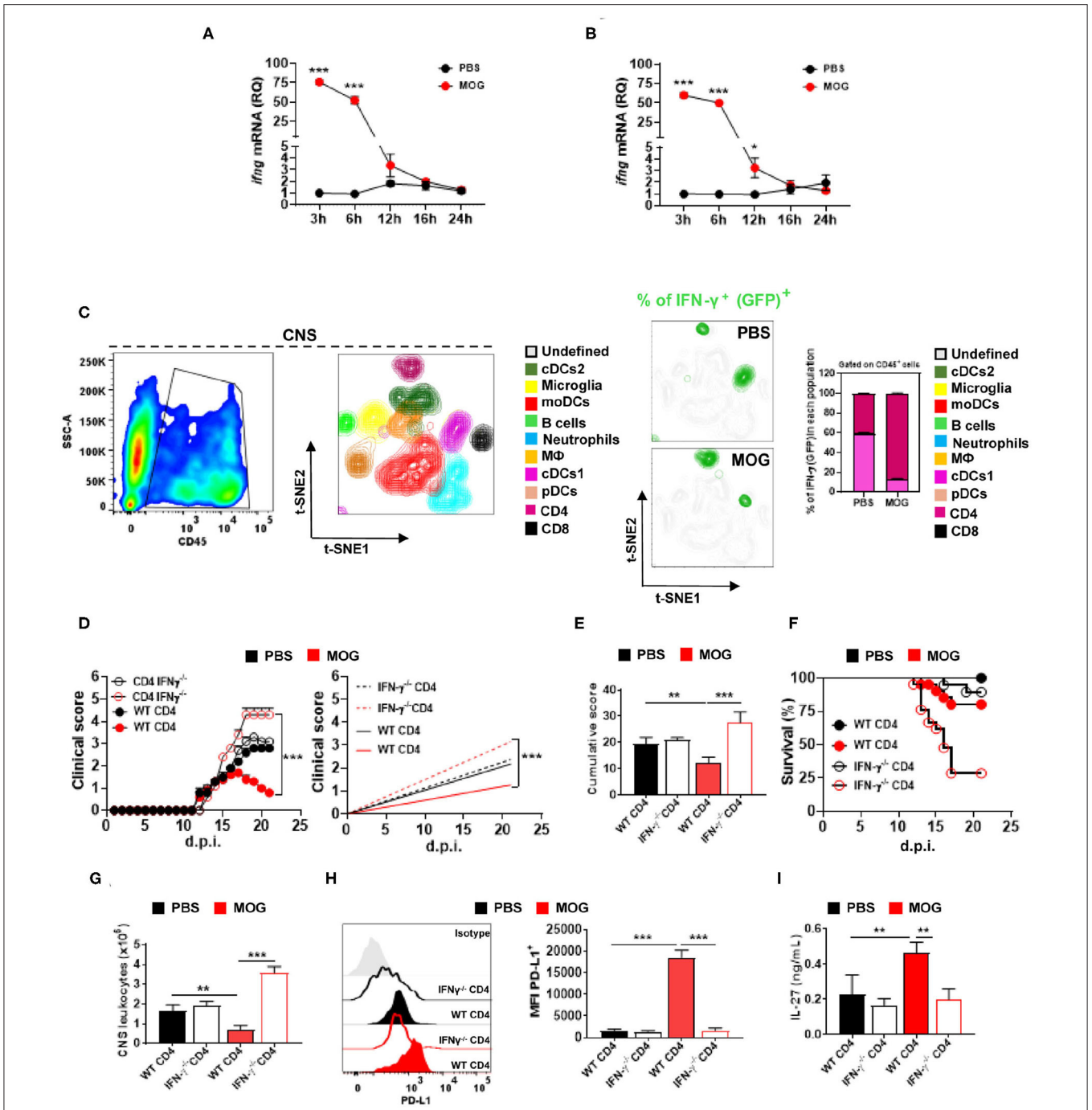


FIGURE 3 | CD4$^+$ T cell-derived IFN-γ is critical for i.v. tolerance induction in EAE. Time course of *Ifng* mRNA, analyzed by qPCR, in the spleen **(A)** and CNS **(B)** after treating mice with EAE i.v. with MOG_{35-55} peptide (200 μg) or PBS ($n = 5$/group in each experiment). **(C)** GFP/IFN-γ reporter mice were immunized for EAE induction and i.v. injected with MOG_{35-55} or PBS at disease onset ($n = 5$/group in each experiment). Mice were sacrificed 3 h post injection and CD45$^+$ cells from the CNS were analyzed by flow cytometry. t-SNE graphs showing the expression and percentage of IFN-γ$^+$ (GFP$^+$) among these cells. Ten populations were identified: microglia (CD11b$^+$ CD45^{low}), monocytes (CD11b$^+$ CD11c$^+$ Ly6c^{high} MHCII^{high} Ly6g$^-$), macrophages (CD11b$^+$ Ly6c^{low} Ly6g$^-$ MHCII$^+$ F4/80^{high}), cDC1 (CD11b$^+$ CD11c$^+$ Ly6c^{med} MHCII^{high} CD26$^+$), cDC2 (CD11b$^+$ CD11c$^+$ Ly6c^{med} MHCII$^+$ CD172α$^+$), pDCs (Lin$^-$ PDCA1^{high}), B cells (CD19$^+$ MHCII$^+$), CD8$^+$ T cells (CD11b$^-$ CD3$^+$ CD8α$^+$), and CD4$^+$ T cells (CD11b$^-$ CD3$^+$ CD4$^+$). **(D)** RAG1$^{-/-}$ mice were reconstituted with 5 × 10^6 total CD4$^+$ T cells from WT or IFN-γ$^{-/-}$ mice ($n =$ 10/group in each experiment); 72 h post reconstitution, recipient mice were immunized for EAE induction and MOG_{35-55} or PBS was given i.v. three times, starting at EAE onset. **(E)** Cumulative disease score for mice shown in **(D)**. **(F)** Survival (%) of EAE mice treated described in **(D)**. **(G)** Mice described in **(D)** were sacrificed 21 d.p.i. and the numbers of CD45$^+$ leukocytes in their CNS were determined by flow cytometry. **(H)** MFI of PD-L1$^+$ moDCs from the CNS of mice shown in **(D)**. **(I)** Splenocytes from mice described in **(D)** were stimulated with MOG_{35-55} for 72 h. Concentrations of IL-27 in culture supernatants were measured by ELISA. All data are representative of at least two experiments, and symbols depict mean ± SEM. Analyses between two groups were carried out by Student's *t*-test, and analyses between four groups by one-way ANOVA with Bonferroni post-test. EAE experiments in **(D)** were analyzed by two-way ANOVA with Bonferroni's multiple comparison. Values of $^*P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ were considered significant.

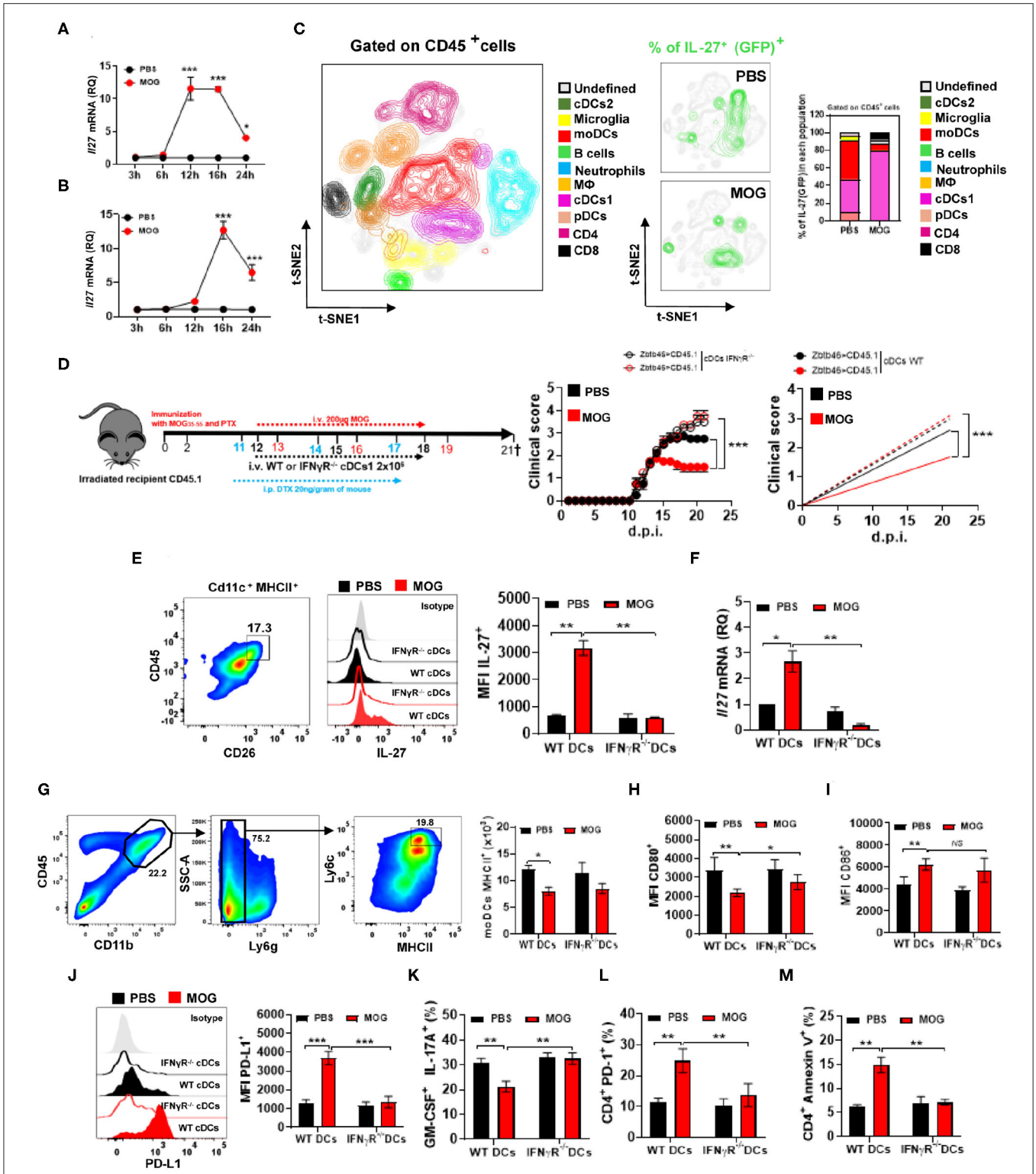


FIGURE 4 | The lack of IFN-γ signaling in cDC1 abrogates their IL-27 production upon i.v. tolerance induction in EAE. Time course of *Il27* mRNA expression analyzed by qPCR, in the spleen **(A)** and CNS **(B)** 24 h after treating mice with EAE i.v. with MOG_{35-55} or PBS ($n = 5$/group in each experiment). **(C)** GFP/IL-27p28 reporter mice were immunized with MOG_{35-55} for EAE induction and i.v. injected with MOG_{35-55} or PBS at disease onset ($n = 5$/group in each experiment). Mice were sacrificed 16 h post injection and cells from the CNS analyzed by flow cytometry. Ten $CD45^+$ cell populations were identified: microglia ($CD11b^+$ $CD45^{low}$), monocytes ($CD11b^+$ $CD11c^+$ $Ly6c^{high}$ $MHCII^{high}$ $Ly6g^-$), macrophages ($CD11b^+$ $Ly6c^{low}$ $Ly6g^-$ $MHCII^+$ $F4/80^{high}$), cDC1 ($CD11b^+$ $CD11c^+$ $Ly6c^{med}$ $MHCII^{high}$ $CD26^+$), cDC2 ($CD11b^+$ $CD11c^+$ $Ly6c^{med}$ $MHCII^+$ $CD172\alpha^+$), pDCs (Lin^- $PDCA1^{high}$), B cells ($CD19^+$ $MHCII^+$), $CD8^+$ T cells ($CD11b^-$ $CD3^+$ $CD8\alpha^+$), and $CD4^+$ T cells ($CD11b^-$

(Continued)

FIGURE 4 | $CD3^+$ $CD4^+$). t-SNE graphs show the expression and percentage of IL-27p28$^+$ (GFP^+) cells. **(D)** $CD45.2^+$ mice were irradiated and transplanted with Zbtb46-iDTR or $CD45.1^+$ BM and 6–8 weeks later immunized with MOG_{35-55} ($n = 7$–10/group in each experiment). cDC depletion (Zbtb46$^+$ MHCII$^+$ CD11c$^+$) was accomplished by i.p. injecting DTX (20 ng/g of mouse weight) every third day after EAE onset. *In vitro* Flt-3-differentiated BMDCs, IFN-γR$\alpha^{-/-}$, or WT was i.v. transferred 1 day post DTX injection, in total twice. MOG_{35-55} or PBS was i.v. injected at 13, 16, and 19 d.p.i. **(E)** At 21 d.p.i, cDC1 (CD11b$^+$ CD11c$^+$ CD45$^+$ Ly6c^{med} MHCII^{high} CD26$^+$) were isolated from the CNS of EAE mice described in **(D)** and their IL-27p28 production was analyzed by flow cytometry and **(F)** by qPCR. The numbers of MHCII$^+$ **(G)**, CD80$^+$ **(H)**, CD86$^+$ **(I)**, and MFI of PD-L1$^+$ **(J)** in moDCs from the CNS of mice described in **(D)**. Frequencies of GM-CSF$^+$ IL-17A$^+$ **(K)**, PD-1$^+$ **(L)**, and annexin V$^+$ **(M)** $CD4^+$ T cells from the CNS of mice described in **(D)**. All data are representative of at least two experiments, and symbols depict mean ± SEM. Analysis between two groups was carried out by Student's *t*-test, whereas analysis between four groups was carried out by one-way ANOVA with Bonferroni post-test. EAE experiments in **(D)** were analyzed by two-way ANOVA with Bonferroni's multiple comparison. Values of $^*P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ were considered significant.

IL-27 production (both mRNA and protein) by CNS IFN-γR$^{-/-}$ cDC1 (**Figures 4E,F**), suggesting that IFN-γ induces IL-27 expression in cDC1. We next investigated whether reduced IL-27 production by IFN-γR$^{-/-}$ cDC1 affected PD-L1 expression by moDCs and encephalitogenic $CD4^+$ T cells. CNS moDCs from IFN-γR$^{-/-}$ cDC1-transplanted mice did not differ in MHCII, CD80, and CD86 expression from moDCs of control mice, but they had significantly fewer PD-L1$^+$ moDCs (**Figures 4G–J**). Failure of tolerance induction in IFN-γR$^{-/-}$ cDC1-transplanted mice was associated with increased frequencies of IL-17$^+$ and GM-CSF$^+$ $CD4^+$ T cells in the CNS (**Figure 4K**) and reduced numbers of annexin V$^+$ and PD-1$^+$ $CD4^+$ T cells (**Figures 4L,M**) compared with WT cDC1-transplanted mice.

We then investigated whether, upon MOG_{35-55}/i.v. injection, CNS and splenic cDC1 uptake MOG_{35-55}, enabling them to directly interact with MOG_{35-55}-specific T cells. Two hours post MOG_{35-55} or PBS i.v. injection into mice with ongoing EAE, cDC1 were FACS-sorted from the CNS (**Supplementary Figures 2B,C**) and co-cultured with 2D2 $CD4^+$ T cells. cDC1 from MOG_{35-55}-injected mice induced greater T cell proliferation and IFN-γ production, compared with cDC1 from PBS-injected mice (**Supplementary Figures 2D–G**). Overall, these data show that cDC1 acquire i.v. injected MOG_{35-55}, which enables them to activate T cells and induce IFN-γ secretion. IFN-γ signaling in cDC1 is required to induce their IL-27 production, PD-L1 expression by moDCs, and i.v. tolerance in EAE.

Interleukin-27 Induces Programmed Death Ligand 1 Expression in Monocyte-Derived Dendritic Cells

Given that IL-27 signaling is critical for i.v. tolerance induction (3) and that *Il27ra*$^{-/-}$ (Wsx-1$^{-/-}$) mice with EAE had reduced numbers of PD-L1$^+$ moDCs in the CNS upon MOG_{35-55}/i.v. treatment, we investigated whether the lack of IL-27 signaling in moDCs affects their PD-L1 expression and tolerance induction. We generated a mixed BM chimeras in which recipient mice (CD45.1) received half BM from *Ccr2*$^{-/-}$ mice and another half from *Il27ra*$^{-/-}$ (CD45.2) mice. In these chimera mice, virtually all monocytes outside of the BM are *Il27ra*$^{-/-}$*Ccr2*$^{+/+}$, as *Ccr2*$^{-/-}$ monocytes fail to leave the BM (37). As a control, we generated mixed chimeras with BMs (1:1) from WT and *Ccr2*$^{-/-}$ mice. Chimera mice were immunized for EAE induction and treated with MOG_{35-55}/i.v. at disease onset. Mice with *Il27ra*$^{-/-}$ BM developed severe disease, compared with mice with WT BM, and MOG_{35-55}/i.v. treatment failed to suppress disease (**Figures 5A,B**). Worsening of disease and treatment failure were associated with a higher number of CNS-infiltrating leukocytes (**Figure 5C**) and greater frequencies of Th1 and Th17 cells, whereas the frequency of apoptotic $CD4^+$ T cells was reduced (**Figures 5D–F**). Moreover, the lack of IL-27 signaling in CNS moDCs precluded upregulation of PD-L1 on them upon MOG_{35-55}/i.v. treatment (**Figure 5G**). Taken together, these data show that MOG_{35-55}/i.v.-induced IL-27 in turn induces PD-L1 expression in CNS moDCs.

CNS and spleen moDCs upregulated PD-L1 under MOG_{35-55}/i.v. treatment, expressing high levels of genes related to Jak–Stat signaling (**Figure 5H**), especially signal transducer and activator of transcription-1 (STAT1) and STAT3. We therefore investigated whether they were involved in IL-27-mediated PD-L1 induction in moDCs. WT, *Stat1*$^{-/-}$, and *Stat3*^{mut} [mutant STAT3 gene with impaired activity (38)] BM-derived DCs (BMDCs) were treated with IL-27 for 24 h, and PD-L1 expression was evaluated. While IL-27 treatment induced PD-L1 expression in WT and *Stat1*$^{-/-}$ moDCs, *Stat3*^{mut} moDCs failed to upregulate PD-L1. Next, when cultured with naive 2D2 T cells, IL-27-treated *Stat3*^{mut} BMDCs were less suppressive to T cell proliferation and GM-CSF production, compared with IL-27-treated WT BMDCs.

To test whether STAT3 is necessary for PD-L1 induction in moDCs *in vivo*, we generated mixed BM chimera mice in which recipient mice (CD45.1) received BM cells from *Ccr2*$^{-/-}$ mice and from *Stat3*^{mut} mice. In these mice, virtually all monocytes and monocyte-derived cells (e.g., moDCs) outside of BM are *Stat3*^{mut}, whereas other immune cells are approximately 1:1 mixture of *Stat3*^{mut} and *Stat3*^{WT} cells. Control chimera mice received BM cells from *Ccr2*$^{-/-}$ and WT mice; in these mice, monocytes and moDCs outside of the BM are *Stat3*^{WT}. Chimera mice were immunized for EAE induction and, after onset of clinical disease, treated with MOG_{35-55}/i.v. Mice with *Stat3*^{mut} BM developed severe EAE and did not respond to MOG_{35-55}/i.v. treatment (**Figures 5I,J**). Treatment failure was associated with an increased number of CNS-infiltrating leukocytes (**Figure 5L**), higher frequencies of CNS-infiltrating Th1 and Th17 cells (**Figure 5M**), reduced frequency of apoptotic $CD4^+$ T cells (**Figure 5N**), and reduced PD-L1$^+$ expression in moDCs (**Figure 5O**). Moreover, although *Ccr2*$^{-/-}$

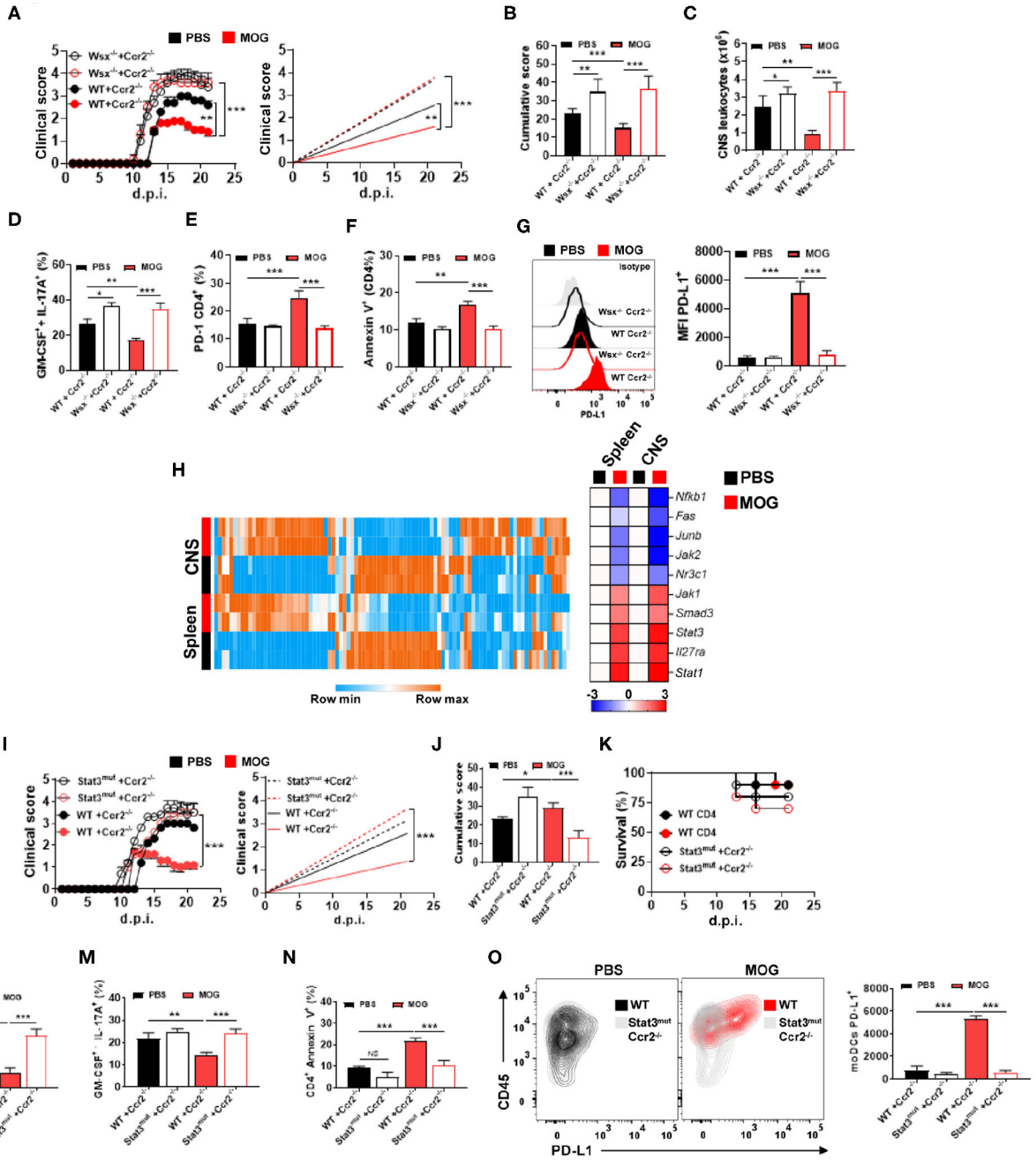


FIGURE 5 | IL-27 induces PD-L1 expression in moDCs *via* STAT3. **(A)** CD45.1$^+$ mice were irradiated and transplanted with 1:1 Wsx$^{-/-}$ and *Ccr2*$^{-/-}$ BM or WT and *Ccr2*$^{-/-}$ BM and 6–8 weeks later immunized with MOG_{35-55} (n = 7–10/group in each experiment). Starting from disease onset, mice were i.v. injected with 200 μg of MOG_{35-55} every 3 days. **(B)** Cumulative disease score for mice shown in **(A)**. **(C)** Mice described in **(A)** were sacrificed at day 21 p.i. and the numbers of CD45$^+$ leukocytes in their CNS were determined by flow cytometry. Frequencies of GM-CSF$^+$ IL-17A$^+$ **(D)**, PD-1$^+$ **(E)**, and annexin V$^+$ **(F)** CD4$^+$ T cells from the CNS of mice shown in **(A)**. **(G)** MFI of PD-L1$^+$ moDCs from the CNS of mice shown in **(A)**. **(H)** Splenic and CNS Ly6c^{high} MHCII$^+$ monocytes were FACS-sorted from WT mice with EAE at 21 d.p.i. and Jak/Stat signaling gene array analysis was performed by qPCR. Heat map showing the expression levels of the top 10 genes. Gene expression levels are row centered, log2 transformed, and saturated at −3 and +3 for visualization. **(I)** CD45.1$^+$ mice were irradiated and transplanted with 1:1 *Stat3*^{mut} and *Ccr2*$^{-/-}$ BM, or WT and *Ccr2*$^{-/-}$ BM, and 6–8 weeks later immunized with MOG_{35-55} (n = 7–10/group in each experiment). Starting from disease onset, mice were i.v. injected with 200 μg of MOG_{35-55} every 3 days. **(J)** Cumulative disease score for mice shown in **(I)**. **(K)** Survival (%) of mice treated as described in **(I)** (n = 10/group in each experiment). **(L)** Mice were sacrificed at day 21 p.i. and the numbers of CD45$^+$ leukocytes from the CNS were determined by flow cytometry. Frequencies of GM-CSF$^+$ IL-17A$^+$ **(M)** and annexin V$^+$ **(N)** CD4$^+$ T cells in the CNS of mice shown in **(I)**. **(O)** Numbers of PD-L1$^+$ moDCs from the CNS of mice shown in **(I)**. All data are representative of at least two experiments, and symbols depict mean ± SEM. Analysis between four groups was carried out by one-way ANOVA with Bonferroni post-test. EAE experiments **(A,I)** were analyzed by two-way ANOVA with Bonferroni's multiple comparison. Values of $^*P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ were considered significant.

Stat3^{mut} chimera mice developed severe EAE, we did not find statistical differences in survival compared with *Ccr2*$^{-/-}$ WT mice (**Figure 5K**). These findings show that IL-27 induces PD-L1 expression in moDCs *via* the STAT3 pathway.

DISCUSSION

Extinguishing harmful immune responses by restoring peripheral tolerance toward auto-Ags has been a long-standing goal in the search for therapies for autoimmune diseases (1, 39). Although depletion of autoreactive T cells and induction of Tregs and tolerogenic DCs are well-known mechanisms of peripheral tolerance (5, 40, 41), our study defines an interplay between molecular and cellular factors that leads to the development of tolerogenic DCs and depletion of autoreactive T cells. Auto-Ag administered i.v. is acquired by cDC1 and presented to auto-Ag-specific T cells, leading to their activation and IFN-γ secretion, which in turn induces IL-27 secretion from cDC1. IL-27 acts on moDCs to induce PD-L1 expression, which then promotes apoptosis of PD-1$^+$ autoreactive T cells and disease amelioration.

It has been established that IFN-γ plays a protective role in EAE through multiple mechanisms (22, 25, 42, 43); it is therefore not surprising that it also mediates disease suppression in i.v. tolerance induction. We have shown that i.v. tolerized mice with EAE have higher frequencies of IFN-γ$^+$ CD4$^+$ T cells compared with controls (3). We show here that i.v. injection of auto-Ag in mice with EAE induces a robust and rapid production of IFN-γ by CD4$^+$ T cells and that blockade of IFN-γ inhibits IL-27 production and PD-L1 expression in CNS moDCs and abrogates tolerance induction. This is in agreement with the findings that IFN-γ prevents accumulation of activated CD4$^+$ T cells in response to Ag stimulation by both inhibiting proliferation and inducing apoptosis of CD4$^+$ T cells (25). Rapid *in vivo*/*in situ* IFN-γ secretion by peptide-specific effector memory CD4$^+$ T cells upon i.v. injection of the peptide has been demonstrated (44), a finding fully applicable to our system. Thus, our results demonstrate that IFN-γ derived from CD4$^+$ T cells is critical for i.v. tolerance induction in ongoing EAE.

Several studies have reported that distinct DC subpopulations can uptake Ags and induce immune tolerance by the induction of IL-27 production and Tregs (3, 10, 45). We have shown that CD11b$^+$ CD103$^-$ DCs are the major source of IL-27 in i.v. tolerance induction in EAE (3). The engagement of IFN-γR on DCs induces their expression of IL-27 and several other regulatory molecules, and IFN-γ-modified DCs modulate EAE severity in an IL-27-dependent manner (42). DCs treated with IFN-γ *in vitro* and injected into mice with EAE suppress disease (46). Consistent with this, we show here that mice lacking IFN-γ signaling in cDC1 fail to recover from EAE upon auto-Ag i.v. treatment. We also show that cDC1 uptake i.v. injected myelin Ag activate CD4$^+$ T cells and their IFN-γ expression and induce tolerance.

The role of monocytes in EAE is viewed as solely pro-inflammatory (47, 48). However, there is evidence that CNS moDCs can acquire regulatory phenotype and facilitate tissue repair (49). Given that IL-27 induces PD-L1 in moDCs (8, 50) and that IL-27 signaling is beneficial in EAE (10, 51), we investigated whether the absence of IL-27 signaling in moDCs affects tolerance induction. We show that the absence of IL-27R in CNS moDCs abrogates the expression of PD-L1 and EAE recovery upon auto-Ag i.v. treatment. In contrast, neutrophils do not upregulate PD-L1 upon injection of auto-Ag; instead, they upregulate PD-L2, which is dispensable for tolerance induction.

It is well-known that IL-27 induces IL-10 expression by T cells and other types of immune cells (10). We and others have shown that i.v. tolerance induction in EAE induces IL-10 production (2, 5), which was also the case in this study. Further, the essential role of IL-10 in i.v. tolerance induction in EAE has been clearly demonstrated (2, 3, 41). We therefore did not test its role again here; however, in future studies, it would be interesting to define a pathway by which IL-10 mediates i.v. tolerance, such as its relevant cellular sources and targets, and to determine which effects of IL-27, if any, are not reliant on IL-10 induction.

Mice lacking PD-L1 develop exacerbated EAE, with PD-L1 on CD11c$^+$ DCs playing an important role in limiting self-reactive CD4$^+$ T cells (52). However, the lack of PD-L2, also a PD-1 ligand, did not worsen EAE (53), demonstrating that PD-L1 has a dominant role in regulating EAE severity. In agreement with these findings, our data reveal that PD-L1 is required for tolerance induction, whereas PD-L2 is dispensable. This is seemingly at odds with studies showing that blockade of PD-L1 with MAb at chronic stage EAE in C57BL/6 mice does not worsen the disease, whereas blockade of PD-L2 does (16, 53). A possible reason for this inconsistency is that we induced i.v. tolerance while clinical disease was still developing, whereas the abovementioned studies started PD-L1 and PD-L2 blockade later, in the chronic phase of disease. Taken together, these findings suggest that the relative importance of cell types and factors they express in regulating disease does change over the disease course.

Studies have shown that IL-27 induces PD-L1 expression (8) and that STAT3, which together with STAT1 mediates IL-27R signaling (3, 10), is required for PD-L1 expression (12, 54). However, the intracellular pathways downstream of IL-27 in i.v. tolerance induction in EAE are still unclear. Consistent with our previous finding that STAT1 is not necessary for IL-27-induced DC modulation (3), we show here that IL-27 from cDC1 induces PD-L1 expression in moDCs *via* STAT3. Indeed, BM chimera mice with impaired STAT3 signaling in moDCs failed to upregulate PD-L1 and to recover from EAE upon MOG_{35-55}/i.v. treatment.

Our findings define the regulatory pathway that suppresses auto-Ag-specific immune response. The prerequisite for activation of this pathway is the existence of a large pool of auto-Ag-specific effector T cells that secrete IFN-γ upon activation with auto-Ag presented by APCs. Injection of a large quantity of auto-Ag induces a burst of IFN-γ secretion from auto-Ag-specific effector T cells, eliciting IL-27 and PD-L1 expression by APCs, which then in turn suppress immune response by causing anergy/apoptosis of the T cells. This is a regulatory feedback mechanism for dampening strong and possibly damaging immune responses. It is likely that this mechanism regulates myelin-specific autoimmune responses in EAE throughout its course, not only after i.v. tolerance induction.

The existence of this pathway provides a unifying explanation for more severe disease in IFN-γ-, IL-27-, and PD-L1-deficient animals. It is likely that additional molecules participate in this pathway, such as IL-10, which is induced in and essential to i.v. tolerance induction in EAE (2, 3, 5, 55); upregulation of TGF-β has also been noted (55), but its significance not explored. Even though we have defined it in the context of i.v. tolerance induction in EAE, this regulatory pathway is certainly relevant in other contexts, being either beneficial or detrimental in them. In addition to $CD4^+$ T cells, the source of IFN-γ could be, for example, pathogen-specific $CD8^+$ T cells or NK cells as well.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Thomas Jefferson University.

AUTHOR CONTRIBUTIONS

GC and BC designed the concept and experiments and wrote the manuscript. GC performed most of the experiments. JR performed some of the flow cytometry experiments and revised the manuscript. RT, HD, AV, LI, and AB performed some *in vivo* experiments and helped with flow cytometry analysis. DH, WZ, and DX revised the manuscript. JP helped with the *in vivo* experiments and revised the manuscript. G-XZ revised the manuscript. JA conducted some *in vivo* experiments and revised the manuscript. AR and BC supervised the studies. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the National Institutes of Health (5R01AI106026) to AR.

ACKNOWLEDGMENTS

The authors would like to thank Katherine Regan for editing the manuscript. They would also like to thank Dr. Ross M. Kedl at the University of Colorado and Dr. Cristopher Hunter at the University of Pennsylvania for providing IL-27p28 reporter mice.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.576752/full#supplementary-material

REFERENCES

1. Serra P, Santamaria P. Antigen-specific therapeutic approaches for autoimmunity. *Nat Biotechnol.* (2019) 37:238–51. doi: 10.1038/s41587-019-0015-4
2. Getts DR, Turley DM, Smith CE, Harp CT, McCarthy D, Feeney EM, et al. Tolerance induced by apoptotic antigen-coupled leukocytes is induced by PD-L1+ and IL-10-producing splenic macrophages and maintained by T regulatory cells. *J Immunol.* (2011) 187:2405–17. doi: 10.4049/jimmunol.1004175
3. Thome R, Moore JN, Mari ER, Rasouli J, Hwang D, Yoshimura S, et al. Induction of peripheral tolerance in ongoing autoimmune inflammation requires interleukin 27 signaling in dendritic cells. *Front Immunol.* (2017) 8:1392. doi: 10.3389/fimmu.2017.01392
4. Bettelli E, Das MP, Howard ED, Weiner HL, Sobel RA, Kuchroo VK. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol.* (1998) 161:3299–306. doi: 10.1016/S0165-5728(98)91500-4
5. Getts DR, Martin AJ, McCarthy DP, Terry RL, Hunter ZN, Yap WT, et al. Microparticles bearing encephalitogenic peptides induce T-cell tolerance and ameliorate experimental autoimmune encephalomyelitis. *Nat Biotechnol.* (2012) 30:1217–24. doi: 10.1038/nbt.2434
6. Wang L, Li Z, Ciric B, Safavi F, Zhang GX, Rostami A. Selective depletion of CD11c(+) CD11b(+) dendritic cells partially abrogates tolerogenic effects of intravenous MOG in murine EAE. *Eur J Immunol.* (2016) 46:2454–66. doi: 10.1002/eji.201546274
7. Batten M, Li J, Yi S, Kljavin NM, Danilenko DM, Lucas S, et al. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. *Nat Immunol.* (2006) 7:929–36. doi: 10.1038/ni1375
8. Casella G, Finardi A, Descamps H, Colombo F, Maiorino C, Ruffini F, et al. IL-27, but not IL-35, inhibits neuroinflammation through modulating GM-CSF expression. *Sci Rep.* (2017) 7:16547. doi: 10.1038/s41598-017-16702-w
9. Fitzgerald DC, Ciric B, Touil T, Harle H, Grammatikopolou J, Das Sarma J, et al. Suppressive effect of IL-27 on encephalitogenic Th17 cells and the effector phase of experimental autoimmune encephalomyelitis. *J Immunol.* (2007) 179:3268–75. doi: 10.4049/jimmunol.179.5.3268
10. Mascanfroni ID, Yeste A, Vieira SM, Burns EJ, Patel B, Sloma I, et al. IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. *Nat Immunol.* (2013) 14:1054–63. doi: 10.1038/ni.2695
11. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev.* (2010) 236:219–42. doi: 10.1111/j.1600-065X.2010.00923.x
12. Diskin B, Adam S, Cassini MF, Sanchez G, Liria M, Aykut B, et al. PD-L1 engagement on T cells promotes self-tolerance and suppression of neighboring macrophages and effector T cells in cancer. *Nat Immunol.* (2020) 21:442–54. doi: 10.1038/s41590-020-0620-x
13. Schreiner B, Bailey SL, Shin T, Chen L, Miller SD. PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T-cell responses in EAE. *Eur J Immunol.* (2008) 38:2706–17. doi: 10.1002/eji.200838137
14. Carter LL, Leach MW, Azoitei ML, Cui J, Pelker JW, Jussif J, et al. PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. *J Neuroimmunol.* (2007) 182:124–34. doi: 10.1016/j.jneuroim.2006.10.006
15. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol.* (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
16. Zhu B, Guleria I, Khosroshahi A, Chitnis T, Imitola J, Azuma M, et al. Differential role of programmed death-ligand 1 [corrected] and programmed death-ligand 2 [corrected] in regulating the susceptibility and chronic progression of experimental autoimmune encephalomyelitis. *J Immunol.* (2006) 176:3480–9. doi: 10.4049/jimmunol.176.6.3480
17. Garnier L, Laffont S, Lelu K, Yogev N, Waisman A, Guery JC. Estrogen signaling in bystander Foxp3(neg) CD4(+) T cells suppresses cognate Th17 differentiation in trans and protects from central

nervous system autoimmunity. *J Immunol.* (2018) 201:3218–28. doi: 10.4049/jimmunol.1800417
18. McCarthy DP, Yap JW, Harp CT, Song WK, Chen J, Pearson RM, et al. An antigen-encapsulating nanoparticle platform for TH1/17 immune tolerance therapy. *Nanomedicine.* (2017) 13:191–200. doi: 10.1016/j.nano.2016.09.007
19. Castro F, Cardoso AP, Goncalves RM, Serre K, Oliveira MJ. Interferon-gamma at the crossroads of tumor immune surveillance or evasion. *Front Immunol.* (2018) 9:847. doi: 10.3389/fimmu.2018.00847
20. Lees JR, Interferon gamma in autoimmunity: a complicated player on a complex stage. *Cytokine.* (2015) 74:18–26. doi: 10.1016/j.cyto.2014.10.014
21. Duong TT, Finkelman FD, Singh B, Strejan GH. Effect of anti-interferon-gamma monoclonal antibody treatment on the development of experimental allergic encephalomyelitis in resistant mouse strains. *J Neuroimmunol.* (1994) 53:101–7. doi: 10.1016/0165-5728(94)90069-8
22. Billiau A, Heremans H, Vandekerckhove F, Dijkmans R, Sobis H, Meulepas E, et al. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol.* (1988) 140:1506–10.
23. Lublin FD, Knobler RL, Kalman B, Goldhaber M, Marini J, Perrault M, et al. Monoclonal anti-gamma interferon antibodies enhance experimental allergic encephalomyelitis. *Autoimmunity.* (1993) 16:267–74. doi: 10.3109/08916939309014645
24. Kroenke MA, Chensue SW, Segal BM. EAE mediated by a non-IFN-gamma/non-IL-17 pathway. *Eur J Immunol.* (2010) 40:2340–8. doi: 10.1002/eji.201040489
25. Chu CQ, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med.* (2000) 192:123–8. doi: 10.1084/jem.192.1.123
26. Ponomarev ED, Novikova M, Yassai M, Szczepanik M, Gorski J, Dittel BN. Gamma delta T cell regulation of IFN-gamma production by central nervous system-infiltrating encephalitogenic T cells: correlation with recovery from experimental autoimmune encephalomyelitis. *J Immunol.* (2004) 173:1587–95. doi: 10.4049/jimmunol.173.3.1587
27. Voorthuis JA, Uitdehaag BM, De Groot CJ, Goede PH, P.H. van der Meide, Dijkstra CD. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. *Clin Exp Immunol.* (1990) 81:183–8. doi: 10.1111/j.1365-2249.1990.tb03315.x
28. Furlan R, Brambilla E, Ruffini F, Poliani PL, Bergami A, Marconi PC, et al. Intrathecal delivery of IFN-gamma protects C57BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. *J Immunol.* (2001) 167:1821–9. doi: 10.4049/jimmunol.167.3.1821
29. Meredith MM, Liu K, Darrasse-Jeze G, Kamphorst AO, Schreiber HA, Guermonprez P, et al. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J Exp Med.* (2012) 209:1153–65. doi: 10.1084/jem.20112675
30. Casella G, Colombo F, Finardi A, Descamps H, Ill-Raga G, Spinelli A, et al. Extracellular vesicles containing IL-4 modulate neuroinflammation in a mouse model of multiple sclerosis. *Mol Ther.* (2018) 26:2107–18. doi: 10.1016/j.ymthe.2018.06.024
31. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science.* (2010) 330:841–5. doi: 10.1126/science.1194637
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* (2001) 25:402–8. doi: 10.1006/meth.2001.1262
33. Mundt S, Mrdjen D, Utz SG, Greter M, Schreiner B, Becher B. Conventional DCs sample and present myelin antigens in the healthy CNS and allow parenchymal T cell entry to initiate neuroinflammation. *Sci Immunol.* (2019) 4. doi: 10.1126/sciimmunol.aau8380
34. Naves R, Singh SP, Cashman KS, Rowse AL, Axtell RC, Steinman L, et al. The interdependent, overlapping, and differential roles of type I and II IFNs in the pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol.* (2013) 191:2967–77. doi: 10.4049/jimmunol.1300419
35. White MPJ, Webster G, Leonard F, La Flamme A.C. Innate IFN-gamma ameliorates experimental autoimmune encephalomyelitis and promotes myeloid expansion and PDL-1 expression. *Sci Rep.* (2018) 8:259. doi: 10.1038/s41598-017-18543-z
36. Giles DA, Duncker PC, Wilkinson NM, Washnock-Schmid JM, Segal BM. CNS-resident classical DCs play a critical role in CNS autoimmune disease. *J Clin Invest.* (2018) 128:5322–34. doi: 10.1172/JCI123708
37. Izikson L, Klein RS, Charo IF, Weiner HL, Luster AD. Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. *J Exp Med.* (2000) 192:1075–80. doi: 10.1084/jem.192.7.1075
38. Steward-Tharp SM, Laurence A, Kanno Y, Kotlyar A, Villarino AV, Sciume G, et al. A mouse model of HIES reveals pro- and anti-inflammatory functions of STAT3. *Blood.* (2014) 123:2978–87. doi: 10.1182/blood-2013-09-523167
39. Steinman L, Ho PP, Robinson WH, Utz PJ, Villoslada P. Antigen-specific tolerance to self-antigens in protein replacement therapy, gene therapy and autoimmunity. *Curr Opin Immunol.* (2019) 61:46–53. doi: 10.1016/j.coi.2019.07.011
40. Li H, Zhang GX, Chen Y, Xu H, Fitzgerald DC, Zhao Z, et al. CD11c+CD11b+ dendritic cells play an important role in intravenous tolerance and the suppression of experimental autoimmune encephalomyelitis. *J Immunol.* (2008) 181:2483–93. doi: 10.4049/jimmunol.181.4.2483
41. Mari ER, Moore JN, Zhang GX, Rostami A. Mechanisms of immunological tolerance in central nervous system inflammatory demyelination. *Clin Exp Neuroimmunol.* (2015) 6:264–74. doi: 10.1111/cen3.12196
42. Murugaiyan G, Mittal A, Weiner HL. Identification of an IL-27/osteopontin axis in dendritic cells and its modulation by IFN-gamma limits IL-17-mediated autoimmune inflammation. *Proc Natl Acad Sci USA.* (2010) 107:11495–500. doi: 10.1073/pnas.1002099107
43. Heremans H, Dillen C, Groenen M, Martens E, Billiau A. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice: enhancement by monoclonal antibodies against interferon-gamma. *Eur J Immunol.* (1996) 26:2393–8. doi: 10.1002/eji.1830261019
44. Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. Visualizing the generation of memory CD4 T cells in the whole body. *Nature.* (2001) 410:101–5. doi: 10.1038/35065111
45. Mari ER, Rasouli J, Ciric B, Moore JN, Conejo-Garcia JR, Rajasagi N, et al. Galectin-1 is essential for the induction of MOG35-55-based intravenous tolerance in experimental autoimmune encephalomyelitis. *Eur J Immunol.* (2016) 46:1783–96. doi: 10.1002/eji.201546212
46. Xiao BG, Wu XC, Yang JS, Xu LY, Liu X, Huang YM, et al. Therapeutic potential of IFN-gamma-modified dendritic cells in acute and chronic experimental allergic encephalomyelitis. *Int Immunol.* (2004) 16:13–22. doi: 10.1093/intimm/dxh003
47. Croxford AL, Lanzinger M, Hartmann FJ, Schreiner B, Mair F, Pelczar P, et al. The cytokine GM-CSF drives the inflammatory signature of CCR2+ monocytes and licenses autoimmunity. *Immunity.* (2015) 43:502–14. doi: 10.1016/j.immuni.2015.08.010
48. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat Neurosci.* (2011) 14:1142–9. doi: 10.1038/nn.2887
49. Giles DA, Washnock-Schmid JM, Duncker PC, Dahlawi S, Ponath G, Pitt D, et al. Myeloid cell plasticity in the evolution of central nervous system autoimmunity. *Ann Neurol.* (2018) 83:131–41. doi: 10.1002/ana.25128
50. Karakhanova S, Bedke T, Enk AH, Mahnke K. IL-27 renders DC immunosuppressive by induction of B7-H1. *J Leukoc Biol.* (2011) 89:837–45. doi: 10.1189/jlb.1209788
51. Do J, Kim D, Kim S, Valentin-Torres A, Dvorina N, Jang E, et al. Treg-specific IL-27Ralpha deletion uncovers a key role for IL-27 in Treg function to control autoimmunity. *Proc Natl Acad Sci USA.* (2017) 114:10190–5. doi: 10.1073/pnas.1703100114
52. Sage PT, Schildberg FA, Sobel RA, Kuchroo VK, Freeman GJ, Sharpe AH. Dendritic Cell PD-L1 limits autoimmunity and follicular T cell differentiation and function. *J Immunol.* (2018) 200:2592–602. doi: 10.4049/jimmunol.1701231
53. Salama AD, Chitnis T, Imitola J, Ansari MJ, Akiba H, Tushima F, et al. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med.* (2003) 198:71–8. doi: 10.1084/jem.20022119
54. Wolfle SJ, Strebovsky J, Bartz H, Sahr A, Arnold C, Kaiser C, et al. PD-L1 expression on tolerogenic APCs is controlled by STAT-3. *Eur J Immunol.* (2011) 41:413–24. doi: 10.1002/eji.201040979

55. Li H, Ciric B, Yang J, Xu H, Fitzgerald DC, Elbehi M, et al. Intravenous tolerance modulates macrophage classical activation and antigen presentation in experimental autoimmune encephalomyelitis. *J Neuroimmunol.* (2009) 208:54–60. doi: 10.1016/j.jneuroim.2009.01.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 January 2021
doi: 10.3389/fimmu.2020.612336

Gut Commensal-Induced IκBζ Expression in Dendritic Cells Influences the Th17 Response

*Lena Michaelis[1], Marcel Treß[1], Hanna-Christine Löw[1], Johanna Klees[1], Christian Klameth[1], Anna Lange[1], Anne Grießhammer[1], Andrea Schäfer[1], Sarah Menz[1], Alex Steimle[1,2], Klaus Schulze-Osthoff[3] and Julia-Stefanie Frick[1]**

[1] Department for Medical Microbiology and Hygiene, Interfaculty Institute for Microbiology and Infection Medicine, University of Tübingen, Tübingen, Germany, [2] Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg, [3] Interfaculty Institute for Biochemistry, University of Tübingen, Tübingen, Germany

OPEN ACCESS

Edited by:
Dipyaman Ganguly, Indian Institute of Chemical Biology (CSIR), India

Reviewed by:
Gianluca Matteoli, KU Leuven, Belgium
Lauren A. Zenewicz, University of Oklahoma Health Sciences Center, United States

***Correspondence:**
Julia-Stefanie Frick
julia-stefanie.frick@med.uni-tuebingen.de

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *30 September 2020*
Accepted: *30 November 2020*
Published: *19 January 2021*

Citation:
Michaelis L, Treß M, Löw H-C, Klees J, Klameth C, Lange A, Grießhammer A, Schäfer A, Menz S, Steimle A, Schulze-Osthoff K and Frick J-S (2021) Gut Commensal-Induced IκBζ Expression in Dendritic Cells Influences the Th17 Response. Front. Immunol. 11:612336. doi: 10.3389/fimmu.2020.612336

Intestinal commensal bacteria can have a large impact on the state of health and disease of the host. Regulation of Th17 cell development by gut commensals is known to contribute to their dichotomous role in promoting gut homeostasis and host defense, or development of autoimmune diseases. Yet, the underlying mechanisms remain to be fully elucidated. One candidate factor contributing to Th17 differentiation, and the expression of which could be influenced by commensals is the atypical nuclear IκB protein IκBζ. IκBζ acts as a transcriptional regulator of the expression of Th17-related secondary response genes in many cell types including dendritic cells (DCs). Insights into the regulation of IκBζ in DCs could shed light on how these immune sentinel cells at the interface between commensals, innate and adaptive immune system drive an immune-tolerogenic or inflammatory Th17 cell response. In this study, the influence of two gut commensals of low (*Bacteroides vulgatus*) or high (*Escherichia coli*) immunogenicity on IκBζ expression in DCs and its downstream effects was analyzed. We observed that the amount of IκBζ expression and secretion of Th17-inducing cytokines correlated with the immunogenicity of these commensals. However, under immune-balanced conditions, *E. coli* also strongly induced an IκBζ-dependent secretion of anti-inflammatory IL-10, facilitating a counter-regulative Treg response as assessed in *in vitro* $CD4^+$ T cell polarization assays. Yet, in an *in vivo* mouse model of T cell-induced colitis, prone to inflammatory and autoimmune conditions, administration of *E. coli* promoted an expansion of rather pro-inflammatory T helper cell subsets whereas administration of *B. vulgatus* resulted in the induction of protective T helper cell subsets. These findings might contribute to the development of new therapeutic strategies for the treatment of autoimmune diseases using commensals or commensal-derived components.

Keywords: dendritic cells, Th17, intestinal commensals, inflammatory bowel disease, immunogenicity, *Escherichia coli*, *Bacteroides vulgatus*, IκBζ

INTRODUCTION

With an area around 200 times larger than the skin, the gastrointestinal mucosa is the largest immunological organ in the body (1). It faces a challenging environment and needs to maintain a careful balance between fighting intestinal intruders and tolerating commensal and nutrition-derived antigens (2). Failure of maintaining gut homeostasis promotes a shift in the microbiota composition, known as dysbiosis and characterized by a loss of bacterial diversity and/or commensals, as well as a bloom of pathobionts (3). A dysbiotic microbiota has been associated with many multifactorial autoimmune diseases such as multiple sclerosis, type 1 diabetes mellitus and inflammatory bowel diseases (IBD) (2, 4).

Dendritic cells (DCs) play a major role in the regulation of gastrointestinal mucosal immunity since they are among the first-line antigen-presenting cells at mucosal surfaces and link the innate and the adaptive immune system (5). DCs encounter a diversity of gut microbes and respond by inducing either immune tolerance to harmless commensal-derived antigens or an inflammatory response to potential pathogens. DCs recognize various surface structures on bacteria, so-called microbe-associated molecular patterns (MAMPs), *via* their patter recognition receptors (PRRs), such as Toll-like receptors (TLRs) (6, 7). Upon sampling of these antigens, DCs undergo a differentiation process resulting in e.g., semi-mature (smDCs) or mature DCs (mDCs), characterized by low or high expression of activation and maturation markers, respectively (8, 9). Under homeostatic conditions, DCs orchestrate the differentiation of naïve $CD4^+$ T cells into functionally distinct T helper cell subsets by creating an environmental cytokine milieu required for the balanced co-existence of regulatory and inflammatory $CD4^+$ T cells (10). In this role, smDCs are known to induce T cell anergy and regulatory T cells (Tregs) whereas mDCs are potent antigen presenting cells promoting $CD4^+$ and $CD8^+$ T cell responses (9).

A subset of IL-17-secreting $CD4^+$ T cells (Th17 cells) plays a dichotomous role in gut homeostasis by promoting protection against fungal and bacterial pathogens on one side, and driving inflammatory pathology and development of autoimmune diseases on the other side (11, 12). The orphan nuclear receptor RORγt is the lineage-determining "master" transcription factor directing the production of the hallmark cytokines IL-17A, IL-17F as well as IL-21 and IL-22 (12, 13). Among these, especially IL-17A plays a dominant role in driving autoimmunity (13). Due to intrinsic instability and plasticity, Th17 cells are able to transdifferentiate to more inflammatory or regulatory phenotypes in response to fluctuating physiological environments (10, 12). Differentiation of Th17 cells is dependent on interleukin 6 (IL-6) and transforming growth factor β (TGFβ), whereas their full maturation depends on IL-1β and IL-23, possibly favoring their pathological activity in the induction of autoimmunity (14, 15). Beyond their demonstrated ability to secrete all these cytokines, how DCs influence plasticity and poise protective and inflammatory responses is not fully known (14).

Besides RORγt, another transcription factor required for Th17 development is the atypical inhibitor of the nuclear factor κB (IκB) protein IκBζ which harbors six ankyrin repeats at its carboxyl terminus, and is encoded by the *Nfkbiz* gene (16, 17). Also known as MAIL or INAP, IκBζ is expressed in a variety of cell types and is essential for the induction of a subset of secondary response genes, e.g., *Il-6*, *Il-12*, *Il-17*, and *Ccl2* (16, 18–20). Transcription of the *Nfkbiz* gene is rapidly induced as primary NFκB response gene upon TLR- and cytokine-receptor signaling (18, 19, 21). The necessity of IκBζ in Th17 development was shown in *Nfkbiz*$^{-/-}$ mice which were resistant to experimental autoimmune encephalomyelitis (EAE), a model of Th17-mediated autoimmune disease with a multiple sclerosis-like phenotype (16, 22).

IκBζ expression in DCs is of particular importance for regulating Th17 development due to the steering role of DCs in states of homeostasis and inflammation. Yet, the impact of a shifting microbiota, as it is observed in states of inflammatory and autoimmune diseases, on IκBζ-dependent, DC-mediated T cell differentiation has not been characterized. In this study, we elucidate the impact of two model gut commensals on the induction of Th17 responses mediated by DCs. We could show that the induction of IκBζ-expression by commensals with low (*Bacteroides vulgatus*) and high (*Escherichia coli*) immunogenicity positively correlates with their immunogenicity in DCs. Furthermore, in an *in vivo* mouse model of IBD, enhancing abundance of these commensals influenced the differentiation of intestinal T helper cells towards rather protective and regulatory phenotypes (*B. vulgatus*) or pro-inflammatory phenotypes (*E. coli*). This effect could experimentally be traced back to the differential expression of IκBζ in DCs.

MATERIALS AND METHODS

Bacteria

Escherichia coli mpk (23) was grown overnight in Luria-Bertani (LB) medium under aerobic conditions at 37°C and subcultivated in the same medium for 2.5 h the next day prior quantification to ensure logarithmic growth phase. *Bacteroides vulgatus* mpk (23) was cultivated in liver broth for 3 days and, prior to quantification, subcultivated in Brain-Heart-Infusion (BHI) medium for 2 days and anaerobic conditions at 37°C to ensure logarithmic growth phase.

Mice

Female C57BL/6NCrl (WT) mice were purchased from Charles River Laboratories. C57BL/6J-Rag1^{tm1Mom} (*Rag1*$^{-/-}$), *TLR2*$^{-/-}$, *TLR4*$^{-/-}$ and *TLR2*$^{-/-}$x*TLR4*$^{-/-}$ mice were obtained from Jackson Laboratories. *Nfkbiz*$^{-/-}$ mice were kindly provided by Dr. M Morimatsu (24) and bred from *Nfkbiz*$^{+/-}$ breeding pairs. For isolation of bone marrow and T cells, 6–12 week old mice were used. All mice were kept and bred under specific pathogen-free (SPF) conditions.

Cultivation and Stimulation of Bone Marrow-Derived Dendritic Cells

Bone marrow cells were isolated and cultivated as described previously (25). At day 7 after isolation, $CD11c^+$ bone marrow derived-dendritic cells (BMDCs) were used for *in vitro* experiments.

1×10^6 BMDCs/ml were stimulated with PBS (mock, Thermo Fisher Scientific), *B. vulgatus* or *E. coli* at a multiplicity of infection (MOI) of 1 at 37°C. 100 ng/ml isolated LPS of *B. vulgatus* [LPS_{BV}, isolated as described in (26, 27) and (28)] or *E. coli* [LPS_{EC}, isolated as described in (26, 27) and (28)] were used for stimulation. For stimulation with a complex microbiota, fecal samples were collected from SPF *Rag1*$^{-/-}$ mice prior to administration of bacteria and induction of colitis, as well as at the end of the experiment (see T cell transfer in *Rag1*$^{-/-}$ mice). Samples were weighed, dissolved in sterile PBS to a stock concentration of 50 mg/ml, heat-inactivated for 15 min at 80°C and filtered through a 100 µm cell sieve. Fecal samples were then further diluted in sterile PBS and BMDCs were stimulated with prepared fecal samples in a concentration of 100 µg/ml. Gentamicin (1 µg/ml) was added to all samples in order to prevent bacterial overgrowth and to create equal treatment conditions. Cells were harvested after the indicated stimulation periods and processed for subsequent analyses.

Cultivation and Stimulation of mICcl2 Cells

Trans-immortalized mouse intestinal epithelial cells derived from the small intestine of a transgenic mouse were cultured as described elsewhere (29). One day prior stimulation, mICcl2 cells were seeded at a concentration of 5×10^5 cells/ml. Cells were stimulated with PBS (mock), *B. vulgatus* or *E. coli* at a MOI of 10 for 2 h and gentamicin (1 µg/ml) was added to all samples in order to prevent bacterial overgrowth and to ensure equal treatment conditions. Cells were gently detached with 0.05% trypsin-EDTA (Gibco) from culture vessels and processed for further analysis.

Isolation of Naïve T Cells

For adoptive T cell transfer and *in vitro* T cell polarization assay, splenic naïve $CD3^+CD4^+CD25^-CD45RB^+$ T cells from female WT mice were purified using a MACS-based negative selection kit (Miltenyi) according to the manufacturer's instructions.

T Cell Polarization Assay

Antigen-independent activation of naïve $CD4^+$ T cells occurred by overnight incubation with plate-bound anti-CD3 (145-2C11) antibodies (BioLegend, coated with 10 µg/ml in PBS) and 2 µg/ml anti-CD28 (37.51) (BioLegend). As polarizing factor, sterile-filtrated cell culture supernatants of 16 h-stimulated BMDCs containing stimulus-dependent cytokine concentrations were used, diluted 1:2 in T cell medium (RPMI 1640 supplemented with 10% FCS, 50 µM 2-mercaptoethanol, 25 mM HEPES, 1% non-essential amino-acids, 1% sodium pyruvate and 1% penicillin/streptomycin). In order to mimic an imbalanced cytokine milieu, neutralizing anti-IL-10 (JES5-2A5) antibodies (BioLegend, 10 µg/ml) were added to naïve T cells simultaneously with the BMDC supernatant. Cells were incubated for 4 h with GolgiStop (BD) prior to end of polarization time and processed for flow cytometry analysis.

Dextran Sodium Sulfate (DSS)-Induced Colitis in WT and *Nfkbiz*$^{-/-}$ Mice

Acute DSS colitis was induced in SPF WT and *Nfkbiz*$^{-/-}$ mice by administration of 2.5% (w/v) DSS (molecular weight 36–50 kDa, MP Biomedicals) dissolved in drinking water for 7 days. Onset of inflammation was assessed on day 0 and on days 3–7 by monitoring body weight and disease activity index (DAI) with parameters ranging from 0–3 regarding blood in stool and on anus, stool consistency, relieving posture and appearance of fur. Colon tissue was used for histopathological analysis by fixing it in 4% formalin and sections stained with Hematoxylin/Eosin (H&E).

T Cell Transfer Colitis in Rag1-/- Mice

Administration of *B. vulgatus* or *E. coli* to 10 week-old *Rag1*$^{-/-}$ mice *via* drinking water in a concentration of 2×10^8 bacteria/ml started one week prior to intraperitoneal injection of $5x10^5$ naïve T cells. Replacing drinking water with bacteria and weighing of mice occurred twice a week. Mice were kept in IVCs in order to maintain stability of the newly developed microbiota composition. Fecal samples were collected prior to administration of bacteria and at the end of the experiment. Mice were sacrificed 5 weeks after T cell transplantation for analysis. Degree of colonic inflammation was determined using colonic histological sections, stained by H&E and scored as described elsewhere (30).

Isolation of Dendritic Cells and T Cells From Colonic Lamina Propria and Mesenteric Lymph Nodes

For isolation of colonic lamina propria (cLP) cells, caecum and colon were thoroughly washed with PBS and cut into 1.5 cm pieces, followed by two incubation periods in HBSS/5% FCS/2 mM EDTA/1 mM DTT, washing in HBSS/5% FCS/1 mM HEPES for 10 min and digestion of minced pieces in RPMI/40 U/ml DNase I/0.12 mg/ml collagenase for 15 min. All steps were performed at 37°C and gentle stirring, with vortexing and filtering through a 100 µM cell strainer in between single steps. Final cell suspension was washed twice with ice-cold HBSS/5% FCS. Immune cells from mesenteric lymph nodes (mLN) were isolated by gentle disruption and passing through a nylon cell strainer (40 µm mesh) with PBS/1% FCS and a washing step with PBS/1% FCS. T cells from cLP and mLN were activated with leukocyte activation cocktail (BD Biosciences) for 4 h with subsequent processing for flow cytometry analysis.

RNA Isolation and RT-PCR

Isolation of RNA from mICcl2 cells, BMDCs and colonic tissue lysates was performed using Qiagen's RNeasy Mini Kit according to manufacturer's instructions. Additional DNA digestion was conducted by using the DNA-free DNA Removal Kit (Thermo Fisher Scientific). SybrGreen based quantitative RT-PCR was performed on a Roche LightCycler480 using Qiagen SybrGreen RT-PCR Kit. Primer annealing occurred at 60°C. 10–100 ng DNase-digested RNA was used for qRT-PCR. Relative mRNA expression in cells stimulated with bacteria to unstimulated cells was determined by using β-actin as housekeeping gene according to the ΔΔCp-method, which takes into account the specific amplification efficiency of every primer pair and each PCR run. Primer sequences: *Nfkbiz* (NCBI Gene ID: 80859) forward: GTGGGAGAACAGATCCGACG, reverse: AGTGAGTGTC

GCTGAACCAG; β-actin (NCBI Gene ID: 11461) forward: CCCTGTGCTGCTCACCGA, reverse: ACAGTGT GGGTGACCCCGTC.

Quantification of Bacteria in Fecal Samples

Plasmid standards were generated by blunt-end cloning using pJET (Thermo Fisher Scientific) and the respective specific 16s PCR fragments of *E. coli* (Primer forward: GTTAATA CCTTTGCTCATTGA, reverse: ACCAGGGTATCTAATC CTGTT (31) or *B. vulgatus* (Primer forward: AACCTGCCG TCTACTCTT, reverse: CAACTGACTTAAACATCCAT (32). The concentration of the isolated plasmids was determined and the standard concentrations were prepared in 10-fold serial dilutions in a range of 100,000–10 copies. Bacterial DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer's instructions. DNA concentration was measured using Qubit dsDNA High Sensitivity Assay (Thermo Fisher Scientific). For the qPCR measurement, DNA concentrations were adjusted to 5 ng per reaction, and PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen). Bacterial copy numbers were determined by a standard curve. For this purpose, log10 of standard copy numbers were plotted against ct-values.

Cytokine Analysis

For determination of IL-6, IL-10, IL-23, IL-1β concentrations in cell culture supernatants, ELISA kits purchased from BD Biosciences or eBiosciences were used according to manufacturers' instructions. For detection of mouse serum cytokines, the Bio-Plex Pro assays Mouse Cytokine 23-Plex and sets for Mouse IL-17F, Mouse IL-21, Mouse IL-22, Mouse IL-23 and TGFβ1 (Bio-Rad) were performed according to manufacturer's instruction and analyzed on a Bio-Plex 200.

Flow Cytometry Analysis

After harvesting or isolation, mICcl2cells, BMDCs, cLP cells and mLN cells were washed and Fc-receptors were blocked for 15 min at 4°C. Staining with fixable viability dyes (Thermo Fisher Scientific) for 15 min at 4°C was applied for live-dead discrimination. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instructions, washed and resuspended in PBS/1% FCS/0.1% saponin. For intracellular staining and cell surface staining, cells were labeled for 30 min at 4°C with fluorophore-conjugated antibodies (all BD, if not stated otherwise) and washed twice. Flow cytometric analyses were performed on a FACS LSRII (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star Inc., USA). Antibodies: CD11c (HL3)-APC, CD11c (HL3)-PE-Cy7, CD4 (RM4-5)-BV605, CD45 (30-F11)-APC-Cy7, CD45R (RA3-6B2)-PE, CD64 (X54-5/7.1)-PE, Foxp3 (MF23)-AF647, Foxp3 (MF23)-BV421, GATA3 (L50-823)-PE-Cy7, IFNγ (XMG1.2)-PE-Cy7, IFNγ (XMG1.2)-APC, IFNγ (XMG1.2)-FITC, IκBζ (LK2NAP)-PerCP-EF710 (Thermo Fisher Scientific), IL-10 (JES5-16E3)-BV510, IL-10 (JES5-16E3)-FITC (BioLegend), IL-17A (TC11-18H10)-PE, IL-17A (TC11-18H10)-APC-Cy7, IL-4 (11B11)-PE, LY6G/C (RB6-8C5)-PE, I-A/I-E (MHC II) (AF6-120.1)-APC, I-A/I-E (MHC II) (AF6-120.1)-BV421, RORγt (Q31-378)-BV421, and T-bet (4B10)-BV421 (BioLegend).

Statistics

Statistical analysis of the data was performed with the GraphPad Prism 8 Software. Data were tested for normality using the Shapiro-Wilk normality test. Statistical analyses were then performed *via* unpaired student's t test or ANOVA for normally distributed data and Mann-Whitney or Kruskal-Wallis multiple comparison test for nonparametric statistics. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Error bars represent + standard deviation (SD).

RESULTS

Expression of IκBζ Promotes Intestinal Homeostasis in a Mouse Model of Acute Colitis

To confirm the role of IκBζ in the modulation of mucosal immune responses, we analyzed the impact of IκBζ-expression on the course of intestinal inflammation in a mouse model of dextran sodium sulfate (DSS) –induced acute colitis. Wild type (WT) and *Nfkbiz*$^{-/-}$ specific-pathogen-free mice were administered 2.5% DSS for seven days in order to induce acute colitis. The severity of disease was estimated by monitoring the weight of the mice and determining the disease activity index (DAI). *Nfkbiz*$^{-/-}$ mice were found to be significantly more susceptible to DSS colitis, as shown by a significantly increased weight loss and DAI, as well as clear signs of severe colitis as shown by histopathological examination of colon sections (**Figure 1**). Based on these results, we conclude that IκBζ plays an important role in maintaining intestinal homeostasis.

IκBζ Expression in BMDCs and Intestinal Epithelial Cells Is Differentially Modulated by Distinct Commensals

Next, we assessed the contribution of two model gut commensals to IκBζ-dependent activation and maturation of DCs. The mouse gut commensal *B. vulgatus* exhibits low immunogenicity and induces smDCs in the colonic lamina propria (cLP), thus contributing to the promotion of homeostasis and prevention of intestinal inflammation in mouse models for colitis (8, 23, 33). *E. coli*, however, is strongly immunogenic and provokes a pro-inflammatory immune response by inducing mDCs, resulting in intestinal inflammation in *Il-2* deficient mice (8, 23, 34). Since bone marrow-derived dendritic cells (BMDCs) are phenotypically similar tointestinal lamina propria DCs (26) and can be generated in high numbers and comparable maturation status, we used BMDCs to evaluate IκBζ induction by *B. vulgatus* or *E. coli*. Wild type (WT) BMDCs were stimulated with either of the two commensals at a MOI of 1 for 16 h, and *Nfkbiz* gene expression as well as IκBζ protein levels were determined at different time points (**Figures 2A, B**, and **Supplementary Figure 1**). *E. coli* stimulation strongly induced *Nfkbiz* gene expression with a maximal

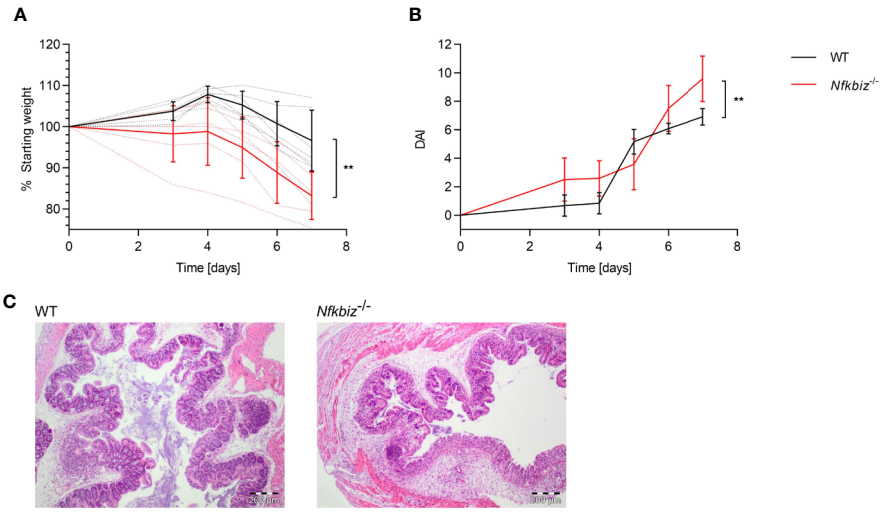


FIGURE 1 | IκBζ expression promotes intestinal homeostasis in the mouse model of DSS colitis. Wild type (WT) (n = 6) and *Nfkbiz*$^{-/-}$ (n = 6) specific-pathogen free (SPF) mice were administered 2.5% DSS (w/v) in drinking water for 7 days to induce colitis. **(A)** Changes in body weight were monitored throughout the experiment: dotted lines indicate each individual, and continuous lines indicate group means ± SD. **(B)** Disease activity index (DAI) was determined according to the criteria mentioned in the material and methods part, with indicated group means ± SD. **p < 0.005 **(C)** Representative H&E stained colon sections.

expression at 2 h post stimulation, followed by a decrease over time to levels close to the starting ones (**Figure 2A**). In contrast, stimulation of WT BMDCs with *B. vulgatus* did not significantly alter the basal levels of *Nfkbiz* gene expression. In agreement with the enhanced mRNA levels, 2 h stimulation of WT BMDCs with *E. coli* strongly increased the IκBζ protein levels in comparison to those in *B. vulgatus*-stimulated BMDCs, which did not differ much from the basal protein levels (**Figure 2B**). Yet, the IκBζ protein levels in *E. coli*-stimulated BMDCs did not decrease as strongly and rapidly as the mRNA levels, indicative of a stable protein. These results suggest that IκBζ expression in BMDCs is differently regulated by commensals, with *E. coli* provoking a strong cell response and *B. vulgatus* a weak one. Hence, the question arises whether IκBζ-mediated cytokine secretion required for T cell polarization is also influenced by commensals.

To address this, we measured the secreted levels of Th17-inducing cytokines IL-6, IL1β and IL-23 as well as anti-inflammatory IL-10 in cell culture supernatants of BMDCs derived from WT and IκBζ-deficient (*Nfkbiz*$^{-/-}$) mice after stimulation (**Figure 2C**). IL-6 and IL-1β are crucial for the induction of RORγt, whereas IL-23 is required for Th17 effector functions, since the receptor for IL-23 (IL-23R) is absent on naïve T cells (35). Upon 24 h-stimulation with *E. coli*, but not with *B. vulgatus*, WT BMDCs secreted significantly higher amounts of IL-6, IL-1β and a clearly higher amount of IL-23 than unstimulated WT BMDCs. In agreement with our previous findings (34), *E. coli* stimulation also significantly enhanced IL-10 secretion by WT BMDCs when compared to stimulation with *B. vulgatus*. Cytokine secretion in *B. vulgatus*-stimulated WT BMDCs was generally very low and did not significantly differ from that in unstimulated WT BMDCs. However, IL-6 and IL-10 secretion by *Nfkbiz*$^{-/-}$ BMDCs stimulated with *E. coli* was significantly lower than that in WT BMDCs, indicating that IL-6 and IL-10 production by BMDCs is dependent on IκBζ. In contrast, deficiency of IκBζ did not significantly reduce cytokine secretion in *B. vulgatus*-stimulated or unstimulated BMDCs. Hence, IκBζ-mediated cytokine secretion by BMDCs seems to be dependent on a strong stimulus, as provided by *E. coli*.

Since DCs are not the only cell type in the gut expressing IκBζ and in direct contact to the microbiota, we also analyzed commensal-mediated effects on IκBζ-expression in mouse intestinal epithelial cells. We stimulated immortalized mouse small intestinal epithelial cells (mICcl2) cells with PBS (mock), *B. vulgatus* or *E. coli* for 2 and 4 h, and measured IκBζ protein and mRNA levels, respectively. Flow cytometry analysis revealed that, similar to what was observed in BMDCs, IκBζ protein levels were significantly higher in *E. coli*-stimulated cells compared to unstimulated or *B. vulgatus*-stimulated cells after 2 h (**Figure 2D** and **Supplementary Figure 2**). After 4 h, a strong induction of *Nfkbiz* gene expression could still be observed in *E. coli*-stimulated, but not *B. vulgatus*-stimulated cells (**Figure 2E**).

These results indicate that commensals display similar immunogenic effects on different cell types of the gut barrier, facilitating a uniform and coordinated immune response by different cell types.

Commensals Trigger Secretion of Th17-Inducing Cytokines in BMDCs *via* TLR4 Signaling

As previously described, immunogenicity of the model commensal bacteria *B. vulgatus* and *E. coli* is mainly mediated by their lipopolysaccharide (LPS) and affects both the maturation status and cytokine secretion of BMDCs (26, 34). To identify the bacterial MAMP and the host TLR responsible for the observed IκBζ induction, *Nfkbiz* gene expression, IκBζ protein and

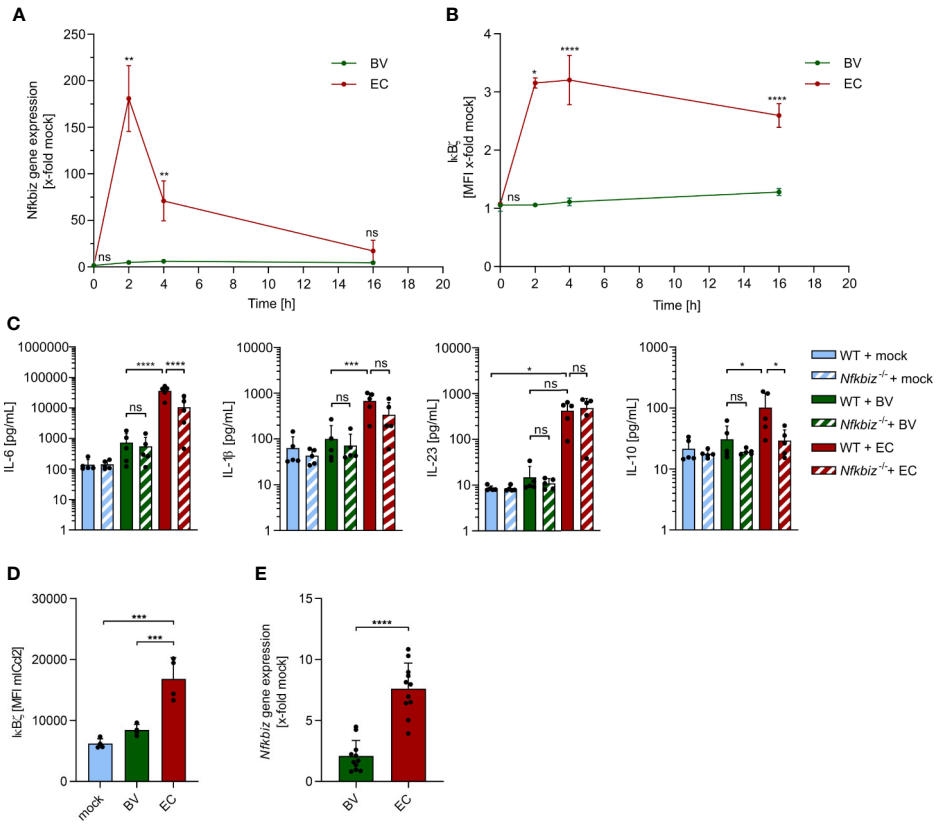


FIGURE 2 | The influence of *B. vulgatus* and *E. coli* stimulation on IκBζ expression in bone marrow-derived dendritic cells (BMDCs) and mICcl2 cells. **(A)** *Nfkbiz* gene expression after stimulation of wild type (WT) BMDCs with *B. vulgatus* (BV) or *E. coli* (EC) at the indicated time points for a total of 16h as determined by RT-PCR (n = 4). **(B)** The respective IκBζ protein levels were determined by flow cytometry analysis. **(C)** Cytokine secretion into cell culture supernatants of WT BMDCs and *Nfkbiz*$^{-/-}$ BMDCs after 24 h stimulation with mock, BV and EC was determined by ELISA (n = 5). **(D)** IκBζ protein levels after 2 h stimulation of mICcl2 cells with mock, BV or EC was determined by flow cytometry and **(E)** *Nfkbiz* gene expression after 4 h stimulation of mICcl2 cells with mock, BV or EC was determined by RT-PCR. Data are represented as geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005.

secreted cytokine levels were determined in stimulated BMDCs isolated from WT as well as TLR2 (*Tlr2*$^{-/-}$), TLR4 (*Tlr4*$^{-/-}$) and TLR2/TLR4 (*Tlr2*$^{-/-}$ × *Tlr4*$^{-/-}$) deficient mice. The significant reduction in *Nfkbiz* gene expression and IκBζ protein levels in *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ × *Tlr4*$^{-/-}$ BMDCs, but not *Tlr2*$^{-/-}$ BMDCs, suggested that the TLR4 ligand LPS was mainly responsible for the high IκBζ induction in *E. coli*-stimulated WT BMDCs (**Figures 3A, B**). In *Tlr2*$^{-/-}$ × *Tlr4*$^{-/-}$ BMDCs, the induced levels were even slightly but not significantly lower than those of single knockouts *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ BMDCs, suggesting a synergistic effect of TLR2 and TLR4 signaling upon strong immunogenic stimulation. Deficiency of TLR2 and/or TLR4 did not significantly influence IκBζ induction in *B. vulgatus*-stimulated BMDCs, emphasizing the low immunogenicity of this commensal. TLR4 signaling was also responsible for the secretion of Th17-inducing cytokines, since the amount of secreted IL-6, IL-1β, IL-23 and IL-10 was significantly reduced in *Tlr4*$^{-/-}$ BMDCs despite a strong stimulus, and slightly but not significantly lower in TLR2 and/or TLR4-deficient BMDCs stimulated with weakly immunogenic *B. vulgatus* (**Figure 3C**).

To evaluate the role of IκBζ in TLR-dependent DC maturation, we correlated the percentage of highly mature BMDCs, as indicated by MHC II^{hi} expression, 16 h post stimulation with the IκBζ protein levels measured 2 h after stimulation in these cells (**Figure 3D**). A positive correlation could be observed which decreased upon deficiency for TLR2 and/or TLR4, suggesting a possible role for IκBζ in TLR-ligand induced maturation processes of BMDCs.

To confirm LPS as the main trigger for IκBζ expression, WT BMDCs were stimulated for 2 h with *B. vulgatus*, *E. coli* and the respective LPS (LPS_{BV} and LPS_{EC}). As expected, IκBζ protein levels normalized to levels in unstimulated BMDCs did not significantly differ between *B. vulgatus* and LPS_{BV} as well as between *E. coli* and LPS_{EC} (**Figure 3E**). Furthermore, LPS_{EC}-induced IκBζ levels were significantly higher than the LPS_{BV}-induced protein levels, mirroring the results obtained with *B. vulgatus* and *E. coli* stimulation. This data suggests that the immunogenicity-dependent effects of *B. vulgatus* and *E. coli* on IκBζ expression, cell maturation and cytokine secretion are mediated by their LPS.

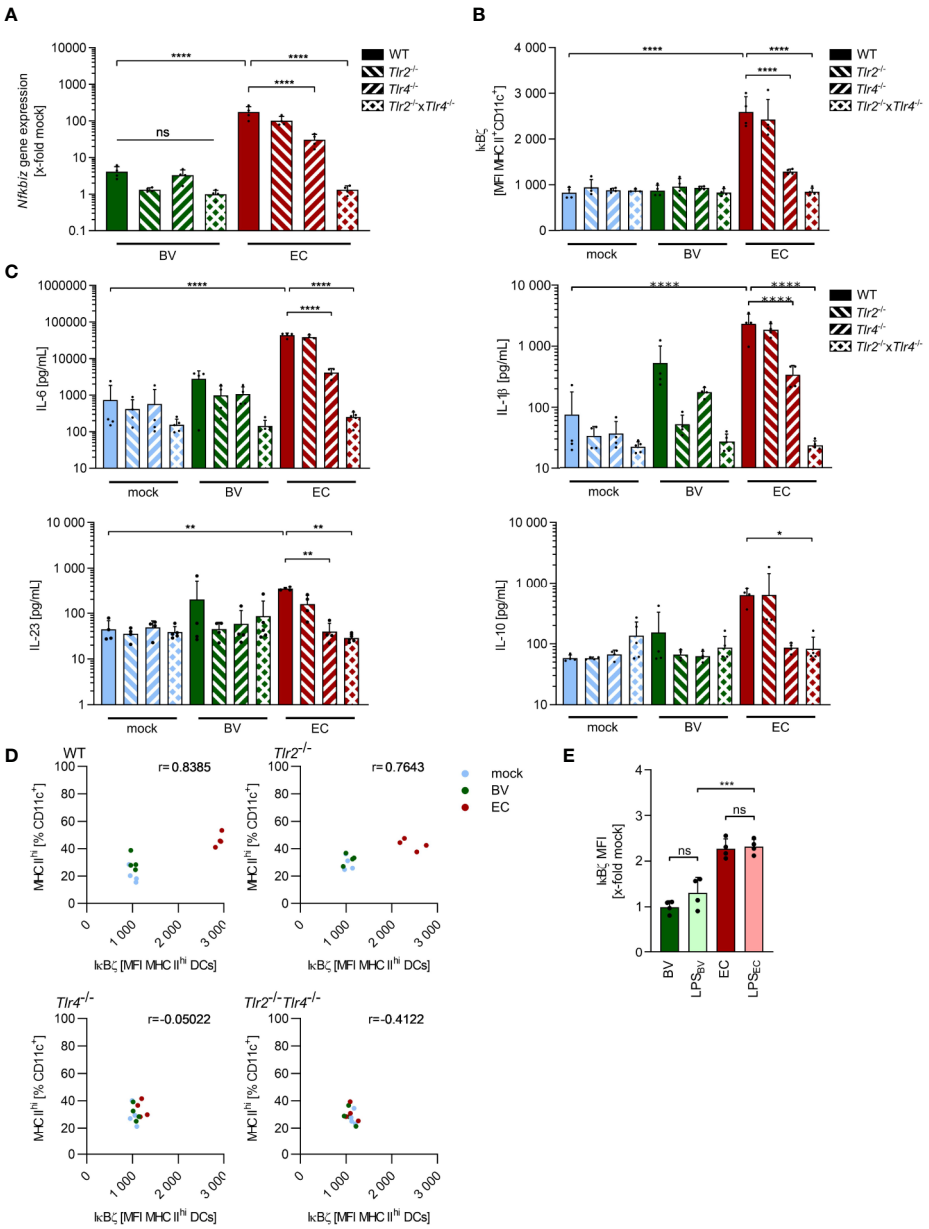


FIGURE 3 | IκBζ induction in bone marrow-derived dendritic cells by commensals is mainly TLR4-dependent. **(A)** *Nfkbiz* gene expression in wild type (WT), *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$x *Tlr4*$^{-/-}$ BMDCs after stimulation with *B. vulgatus* (BV) or *E. coli* (EC) as determined by RT-PCR. **(B)** The respective IκBζ protein levels were determined by flow cytometry after 2 h of stimulation. **(C)** Cytokine secretion into cell culture supernatants after 16 h of stimulation was determined by ELISA. **(D)** Correlation between percentages of MHC II^{hi}-expressing BMDCs after 16 h of stimulation and the IκBζ levels after 2 h with indicated Pearson r **(E)** IκBζ levels in WT BMDCs after stimulation with BV, LPS_{BV} (100 ng/ml), EC, LPS_{EC} (100 ng/ml) for 2 h, normalized to IκBζ levels in unstimulated WT BMDCs. Data represent geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005.

The Unique Composition of the Cytokine Milieu in Response to Various Commensals Differentially Polarizes T Cells

Antigen-inexperienced, i.e., naïve, CD4$^+$ T cells can differentiate into multiple lineages upon activation, depending on the local environment mainly defined by the composition and concentration of the available cytokines (36, 37). As we observed a distinct cytokine secretion pattern in response to *B. vulgatus* and *E. coli*, we analyzed the influence of the different cytokine milieu on CD4$^+$ T cell differentiation. To this aim, we antigen-independently activated naïve CD4$^+$ T cell with plate-bound anti-CD3 and soluble anti-CD28, and defined their polarization fate in response to sterile-filtrated cell culture supernatant (SN) of BMDCs previously stimulated for 16 h

with PBS (mock SN), *B. vulgatus* (BV SN), or *E. coli* (EC SN) (**Figure 4A** and **Supplementary Figure 3**). As control, differentiation in presence of the sole BMDC medium was performed.

To mimic an imbalance between pro- and anti-inflammatory cytokines as reported for the pathogenesis of autoimmune diseases (38), we added neutralizing anti-IL-10 antibody to the cell culture supernatants (medium + anti-IL-10, mock SN+ anti-IL-10, BV SN + anti-IL-10, EC SN + anti-IL-10). Neutralization of extracellular IL-10 appeared to slightly reduce T cell survival after 72 h of incubation (**Figure 4B**). Yet, it also induced a more pronounced differentiation of naïve T cells into Th1 (IFNγ+CD4$^+$) and Th2 (IL-4$^+$CD4$^+$) effector helper subsets. Furthermore, it significantly increased differentiation into Th17 cells (IL-17$^+$ CD4$^+$) when present alone (BMDC medium only) and in combination with BV SN or EC SN. Yet, BV SN and EC SN induced similar levels of Th17 cells, under both balanced and imbalanced cytokine conditions.

Th17 cells are known to have certain plasticity. On the one hand, they are able to convert to Th1-like Th17 cells, co-expressing IL-17 and IFNγ, and contributing to increased inflammatory activity (39). On the other hand, anti-Th17 Treg cells co-expressing IL-17 and Foxp3 were shown to suppress CD4$^+$ T cell proliferation, and found in the inflamed intestinal mucosa of patients with Crohn´s Disease (40). Th17 cells co-expressing IL-17 and IL-10 are instead protective and prevent the accumulation and activity of inflammatory Th17 at sites of inflammation (41). Therefore, we further characterized differentiated Th17 cells with respect to the co-expression of IL-17 with IFNγ, Foxp3 or IL-10 to define their inflammatory or non-inflammatory potential. No significant influence of the differentiation environments on the subsets of Th17 cells was observed (**Figure 4B**, bottom panels). However, the percentage of Foxp3$^+$ IL-17$^+$ T cells and IFNγ$^+$ IL17$^+$ T cells significantly increased upon neutralization of IL-10 in BV SN, suggesting that, even in absence of anti-inflammatory IL-10, a balanced Th17 immune response is guaranteed by an increased number of anti-Th17 Tregs.

E. coli Promotes a Pro-Inflammatory CD4$^+$ T Cell Response in the Mouse Model of T Cell Transfer Colitis

The initial lack of Tregs and induction of inflammatory Th1 and Th17 cells are known to play a role in disease onset in the T cell transfer model of colitis in *Rag1*$^{-/-}$ mice (42, 43). Transfer of naïve T cells into these immune-deficient mice lacking functional T cells and B cells induces a chronic colonic inflammation that is largely dependent on the microbiota composition (44).

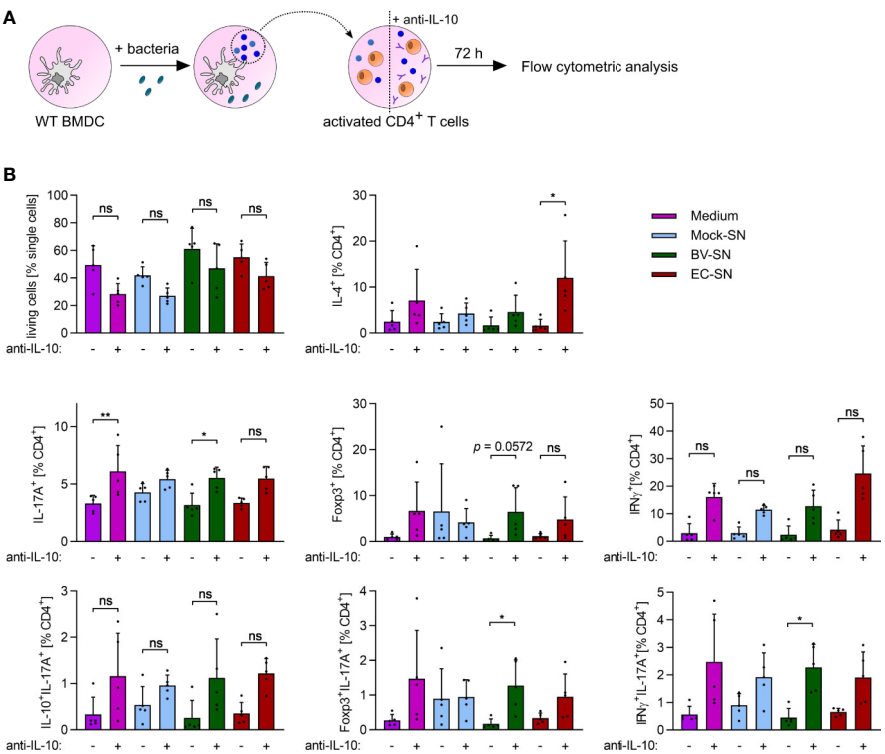


FIGURE 4 | *In vitro* CD4$^+$ T cell polarization in response to the supernatant of BMDCs. **(A)** Overview of the experimental setup: WT BMDCs were stimulated with PBS (mock), *B. vulgatus* (BV) or *E. coli* (EC) for 16 h. The resulting supernatants were sterile-filtrated and used for differentiation of anti-CD3/anti-CD28-activated WT CD4$^+$ T cells with or without addition of neutralizing anti-IL-10 antibodies (10 µg/ml). After 72 h, T cells were analyzed by flow cytometry. **(B)** Flow cytometry analysis of differentiated CD4$^+$ T cell subsets after incubation with T cell medium or supernatants of mock, BV or EC-stimulated WT BMDCs with or without addition of neutralizing anti-IL antibodies (10µg/ml) (n = 5). Data represent geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005.

We therefore analyzed the impact of administration of a symbiont or a pathobiont, respectively, on DC responses and T helper cell polarization in the colonic lamina propria (cLP) and mesenteric lymph nodes (mLN). SPF *Rag1*$^{-/-}$ mice were administered either *B. vulgatus* or *E. coli* by continuous administration of 2×10^8 bacteria per mL drinking water, starting one week prior to transplantation of 5×10^5 naïve T cells (**Figure 5A**). Mice were weighed and drinking water renewed twice a week. Mice were sacrificed five weeks after T cell transplantation.

Administration of *B. vulgatus* or *E. coli* did not lead to significant differences in weight loss over time (**Figure 5B** and **Supplementary Figure 4**). However, a slightly accelerated weight loss was observed in *E. coli*-administered mice starting three weeks after T cell transplantation compared to mice administered with either *B. vulgatus* or no bacteria. Furthermore, a high variation within experimental groups was observed, as indicated by large standard deviations in **Figure 5B**. To evaluate the influence of *B. vulgatus*- and *E. coli*-administration on systemic inflammation, the concentration of serum cytokines was determined (**Figure 5C**). No significant differences were observed between the different experimental groups with the exception of increased IL-21 levels in some *E. coli*-administered mice. Serum concentrations of Th17-inducing IL-6 and IL-1β were very low with IL-23 concentrations even under the detection limit in all mice. Concentrations of the anti-inflammatory IL-10 did positively correlate with concentrations of many pro-

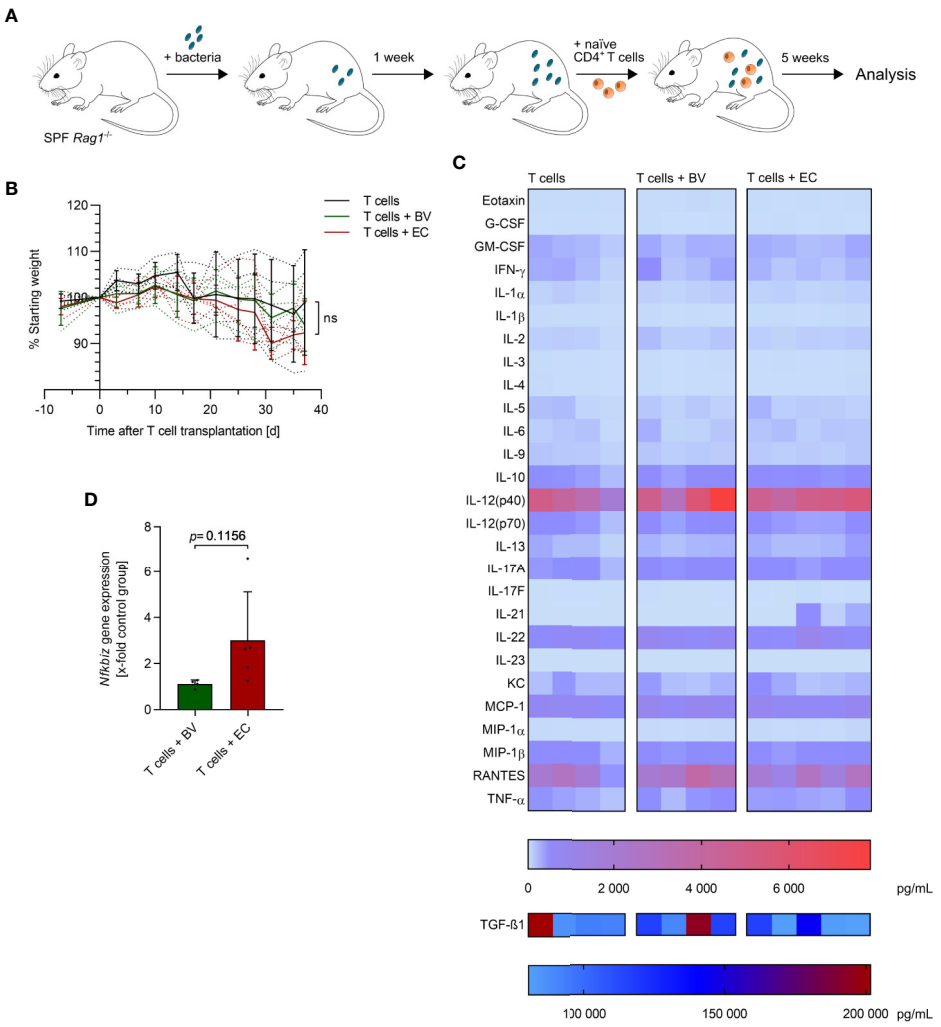


FIGURE 5 | *E. coli*-administration accelerates colitis induction in a T cell transfer model of colitis. **(A)** Overview of the experimental setup: specific-pathogen free (SPF) *Rag1*$^{-/-}$ mice were continuously administered with *B. vulgatus* (n = 4) or *E. coli* (n = 5) *via* drinking water. A control group was left untreated (n = 4). After one week of bacterial association, naïve CD4$^+$ T cells were transplanted. Mice were sacrificed 5 weeks after T cell transplantation. **(B)** The change in bodyweight was monitored throughout the experiment: dotted lines indicate each individual and continuous lines indicate group means ± SD, ns, not significant. **(C)** The concentration of the indicated cytokines was determined in mouse serum, using Bio-Plex assays. Each column represents one individual. **(D)** RNA was isolated from colonic tissue and *Nfkbiz* gene expression was determined by RT-PCR. Data represent geometric mean + SD.

inflammatory cytokines (**Supplementary Figure 5**), indicating a systemic repressive function. Yet, *Nfkbiz* gene expression in colonic tissue was found to be higher upon *E. coli*-administration, compared to *B. vulgatus*-administration, giving first hints of a more pronounced Th17 response to a microbiota rich in *E. coli* (**Figure 5D**).

Flow cytometry analyses revealed that total numbers of cLP DCs were significantly higher in the *B. vulgatus*-administered group compared to the control group, whereas *E. coli*-administration resulted in only slightly increased numbers (**Figure 6A** and **Supplementary Figure 6**). A positive correlation between the maturation status of cLP DCs, as indicated by MHC II^{hi} expression, and IκBζ protein levels in these DCs was observed, with only low percentages of highly mature DCs in *B. vulgatus* or *E. coli*-administered mice (**Figure 6B**, left panel). However, highly mature DCs with low IκBζ levels were observed in mLN of *B. vulgatus* or *E. coli*-administered mice (**Figure 6B**, right panel). In the control group, the percentage of $IκBζ^{hi}$ MHC II^{hi} mLN DCs remained low.

Total numbers of recruited cLP $CD4^+$ T cells were not found to be dependent on microbiota composition (**Figure 6C**). Yet, the polarization of these $CD4^+$ T cells seemed to be conditioned by microbiota composition: enhanced abundance of *B. vulgatus* clearly induced more Tregs than *E. coli*-administration or no microbiota-manipulation. Also the total numbers of $Foxp3^+$ $IL\text{-}17^+$ anti-Th17 Tregs were significantly higher, and total numbers of $IL10^+$ $IL\text{-}17^+$ protective Th17 cells were slightly higher in *B. vulgatus*-administered mice compared to the other groups. In contrast, *E. coli*-administration resulted in significantly higher total numbers of Th1 cells and slightly higher total numbers of Th1-like Th17 cells than the control group, but at levels similar to those detected in the *B. vulgatus*-administered group. Total numbers of Th2 cells were significantly higher in *B. vulgatus*- or *E. coli*-administered groups compared to the control group. Taken together, polarization of $CD4^+$ T cells in the cLP is tilted to rather anti-inflammatory and regulatory phenotypes in *B. vulgatus*-administered mice whereas in *E. coli*-administered mice, cLP $CD4^+$ T cells express rather pro- inflammatory markers, promoting colonic inflammation.

In order to evaluate the role of DCs in the induction of the observed phenotypes and overall disease progression, we correlated IκBζ-expression in cLP DCs with $CD4^+$ T cell phenotypes in cLP and mLN (**Figure 6D**). IκBζ-expression in cLP DCs was negatively correlated with total numbers of cLP Th1 cells in all experimental groups (**Figure 6D**, left panel). Furthermore, induction of the anti-Th17 Tregs seemed to be dependent on intermediate IκBζ-levels as observed in the *B. vulgatus*- administered group (**Figure 6D**, right panel). High and low IκBζ-levels in cLP DCs in control or *E. coli*-administered mice did not correlate with high numbers of anti-Th17 Tregs.

With respect to the classical Th1/Th2 balance, a shift towards autoimmune-disease promoting Th1 cells was observed in *E. coli*-administered mice whereas in *B. vulgatus*- administered mice the number of Th2 cells exceeded the number of Th1 cells (**Figures 6C, E**) (45). Consistently, the grade of colonic inflammation negatively correlated with the induction of protective Th17 cells in mLN, emphasizing the anti-inflammatory role of these cells (**Figure 6F**).

Increased Abundance of *B. vulgatus* in Microbiota Dampens the Secretion of Pro-Inflammatory Cytokines by BMDCs

The above presented results indicate that the enhanced intestinal abundance of *B. vulgatus* leads to an increase in regulatory/anti-inflammatory $CD4^+$ T cell subsets whereas higher numbers of *E. coli* promote differentiation of pro-inflammatory $CD4^+$ T cells in an immune-compromised host with a presumably dysbiotic microbiota. However, a differential activation of DCs by the two commensals could not be clearly observed in these mice. To directly link our *in vitro* results with those obtained in the model of T cell transfer colitis, we collected fecal samples from representative *Rag1*$^{-/-}$ mice with a presumably dysbiotic microbiota (DYS) prior to bacterial administration and from T cell transplanted mice with or without commensal enrichment after development of colitis (DYS + TC, DYS + TC + BV, DYS + TC + EC). Heat-inactivated fecal samples were then used to stimulate WT BMDCs (**Figure 7A**). Increased abundance of *E. coli* induced significantly higher *Nfkbiz* gene expression (**Figure 7B**) and IκBζ–protein levels (**Figure 7C**) than all the other microbiota. However, we did not observe a significant increase in the secretion of pro-inflammatory cytokines in response to DYS + TC + EC- stimulation as compared to DYS+TC (**Figure 7D**). Rather, 4 h stimulation of BMDC with DYS + TC + BV decreased the secretion of pro-inflammatory cytokines, with a slight but not significant decrease of secreted IL-6, and a significant lower secretion of IL-23 compared to DYS + TC or DYS + TC + EC. The levels of IL-10 decreased as a result of T cell transfer.

DISCUSSION

The impact of the intestinal microbiota on health and disease is indisputably large. Due to the close link between microbiota and host immunity, it is not surprising that dysbiosis is associated with many diseases linked to a malfunctioning immune system, e.g., autoimmune diseases. A local impact of a disturbed microbiota is well described for inflammatory bowel diseases (IBD) such as Crohn's disease or ulcerative colitis). Moreover, many extra-intestinal diseases such as type 1 diabetes, rheumatoid arthritis, asthma or multiple sclerosis have been reported to be influenced by the microbiota (3, 46).

The first model commensal used in this study, *B. vulgatus*, belongs to the phylum of Bacteroidetes, one of the most abundant phyla in the mammalian gut, and was found to reduce inflammation in mouse models of colitis (23, 47). A decrease in *Bacteroides* species was reported in patients with IBD, together with the simultaneous increase of facultative anaerobes such as *E. coli*, the second model commensal used in this study (48). *E. coli* is a colitogenic pathobiont,that can promote intestinal inflammation in genetically predisposed hosts (23). Differences in bacterial immunogenicity as well as in the interaction with several cell types of the innate and adaptive

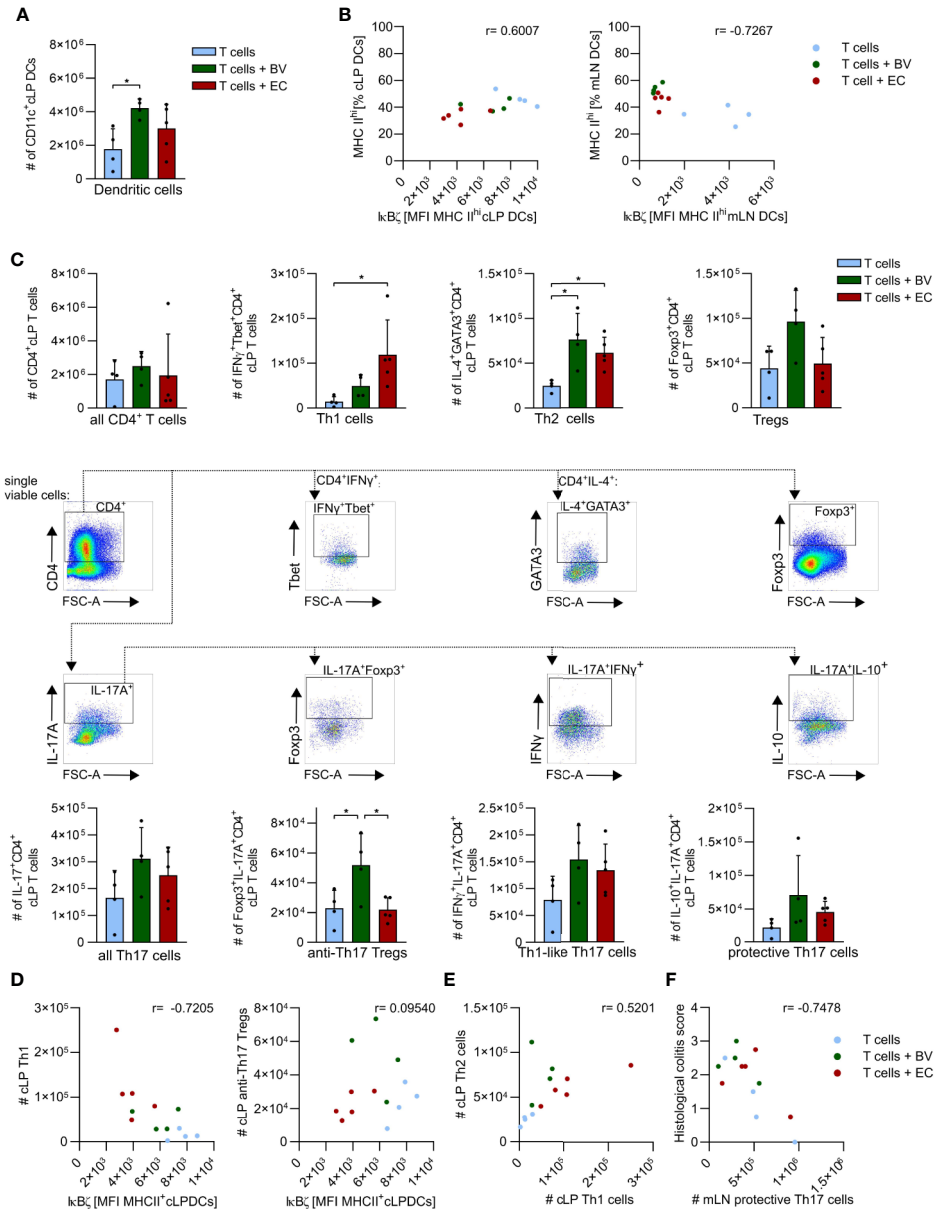


FIGURE 6 | *B. vulgatus* promotes induction of immune regulative mechanisms in a T cell transfer model of colitis. Dendritic cells (DCs) and CD4$^+$ T cells were isolated from the colonic lamina propria (cLP) and mesenteric lymph nodes of T cell transplanted *Rag1*$^{-/-}$ mice and analyzed by flow cytometry (see Fig.5) **(A)** Total number of cLP CD11c$^+$ DCs as determined by flow cytometry. **(B)** Correlation between percentages of MHC II^{hi} expressing cLP (left panel) and mLN DCs (right panel) and their IκBζ levels, indicated Pearson r. **(C)** CD4$^+$ T cell phenotypes in cLP with corresponding gating. **(D)** Correlations between IκBζ expression in cLP DCs and cLP Th1 cells or CLP anti-Th17 Tregs are indicated Pearson r. **(E)** Th1/Th2 balance in cLP. **(F)** Correlation between histological colitis score and number of cLP protective Th17 cells, as indicated Pearson r. Data represent geometric mean + SD, *p < 0.05.

immune system are accountable for these contrary outcomes. In this study, we focused on the interaction of commensals with DCs and the resulting CD4$^+$ T cell response *in vitro* and *in vivo* in an autoimmune-driven mouse model of colitis (49).

The role of IκBζ has already been extensively studied in various autoimmune diseases and cell types. For instance, keratinocyte-derived IκBζ was found to drive psoriasis (50, 51); and mice deficient in IκBζ are resistant to EAE due to a defect in Th17 development, explainable by the fact that IκBζ enhances IL-17 expression by directly binding to the regulatory region of the *Il-17* gene (16). However, IκBζ-deficient epithelial cells provoke a Sjögren's syndrome-like inflammation in mice, and IκBζ-deficient hepatocytes showed defective proliferation due to impaired TLR4-signaling (21, 52). Furthermore, IκBζ exerts both inhibitory and transcription-promoting effects on NFκB activity. The transcription factor NFκB plays an

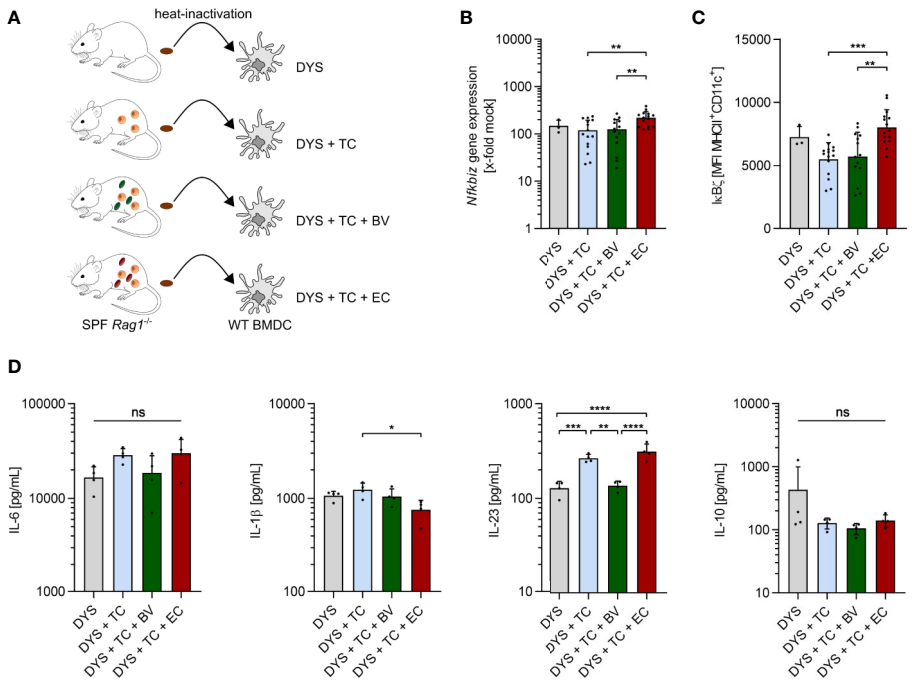


FIGURE 7 | Enhanced abundance of *E. coli* increases IκBζ levels in dendritic cells. **(A)** Overview of the experimental setup: fecal samples were collected from SPF *Rag1*$^{-/-}$ mice prior to bacterial administration and T cell transplantation (DYS) as well as from T cell transplanted mice with established colitis, and left untreated (DYS + TC) or administered with *B. vulgatus* (DYS + TC + BV) or *E. coli* (DYS + TC + EC) *via* drinking water. Samples were dissolved in PBS, heated for 15 min at 80°C, filtered and used in a concentration of 100 µg/ml for a 2 h and 4 h stimulation of wild type (WT) bone marrow-derived dendritic cells (BMDCs). **(B)** *Nfkbiz* gene expression, as determined by RT-PCR and **(C)** IκBζ protein levels, as determined by flow cytometry, after 2 h stimulation of WT BMDCs. **(D)** Secreted cytokines after 4 h stimulation of WT BMDCs, as determined by ELISA. Data represent geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005.

importantrole in cellular responses to stress, injury and inflammation (53). Its subunits p50, p52, p65 (RelA), RelB and c-Rel can form various homo- and heterodimers which bind to specific DNA elements to induce target gene expression of e.g., IL-6, IL-1β, IL-23, or IL-10 (54–56). More recently, upregulation of *Nfkbiz* has been detected in inflamed intestinal tissue of UC patients, suggesting that an altered function of IκBζ may contribute to the development of the disease (57). We could demonstrate a deleterious effect of IκBζ deficiency in the mouse model of acute DSS-induced colitis (**Figure 1**). *Nfkbiz*$^{-/-}$ mice progressed towards a significantly more severe disease than WT mice, indicating an important role of IκBζ for intestinal inflammatory responses to DSS administration. Taken together, these data suggest that the function of IκBζ needs to be tightly regulated in many cell types: too much or too little of its activity can lead to disease.

We could also show that bacterial immunogenicity regulates IκBζ expression in DCs thus driving either an inflammation-promoting or tolerogenic DC phenotype. In our previous studies, we demonstrated that *B. vulgatus* induces smDCs, characterized by a lower expression of maturation markers, such as MHC II, CD40, CD80 and CD86, as well as lower secretion of pro-inflammatory cytokines compared to mDCs induced by *E. coli* (8, 58). These smDCs are tolerant towards maturation-inducing stimuli and are unable to induce pro-inflammatory Th1 and Th17 responses (8). Here, we could relate the expression levels of IκBζ to the degree of DC maturation and induction of T cell differentiation: *E. coli*, but not *B. vulgatus* increased the mRNA and protein levels of IκBζ (**Figures 2A, B**). Based on this, we propose that the transition from smDCs to mDCs requires a relief of the tight regulation on IκBζ expression and activity.

IκBζ is generally induced by stimulation with MAMPs or the cytokines IL-1, IL-17 and IL-18 (16, 20, 21, 57). Here, we could demonstrate that *E. coli*-induced IκBζ expression as well as BMDC maturation is mainly mediated by LPS *via* TLR4 signaling (**Figure 3**). Despite being one of the most conserved structures in Gram-negative bacteria, differences in immune-activating activities of LPS have been observed before: isolated LPS_{BV} displayed only weak agonistic interactions with the host MD2/TLR4 receptor complex, thus inducing smBMDCs, whereas isolated LPS_{EC} potently activated the MD2/TLR4 receptor complex, causing rather pro-inflammatory signaling by mBMDCs (26). We additionally demonstrate that the extent of LPS-induced TLR4 signaling impacts the ability of BMDCs to induce a Th17 response: a stronger activation significantly enhances secretion of Th17-promoting cytokines by BMDCs (**Figures 2C** and **3C**). In addition, we observed a synergistic effect of TLR2 and TLR4 signaling upon a strong stimulus, indicated by a decreased response in BMDCs deficient for both receptors compared to BMDCs deficient for only one of these TLRs. This observation supports earlier findings, describing a marked increase in pro-

inflammatory cytokine secretion by mouse peritoneal macrophages upon co-stimulation with TLR2 and TLR4 ligands compared to the stimulation of either receptor alone (59). As previously reported, only secretion of IL-6 and IL-10 was found to be IκBζ-dependent (21, 60). IL-6 is mainly induced by p65/p50 NFκB heterodimers and IL-10 by p50/p50 NFκB homodimers (56, 61). IκBζ preferentially associates with p50 present in p65/p50 heterodimers or p50/p50 homodimers, stabilizes promoter binding and thus assists expression of IL-10 and IL-6 (17, 60, 61). IL-1β is mainly induced by subunits p65 and cRel and, thus, presumably not preferentially bound by IκBζ (62). Nevertheless, an indirect influence of IκBζ activity on IL-1β secretion has been elucidated recently: IκBζ upregulates the transcription of the *Nlrp3* gene, which encodes the inflammasome component NLRP3 (63). Activation of the NLRP3 inflammasome leads to the cleavage of inactive pro-IL-1β into active IL-1β, which can then be secreted by the cell. Kim et al. reported that *Nfkbiz* deficiency results in impaired IL-1β secretion, which we could confirm (**Figure 2C**). An IκBζ-dependent regulation of IL-23 secretion by BMDCs was however not observed.

We tested whether the cytokine milieu of stimulated BMDCs is sufficient for determining the differentiation fate of already activated T cells. We could not observe significant induction of effector T cells (Th1, Th2, Tregs, Th17 subsets) (**Figure 4**). A slightly but insignificantly increased survival of T cells could be observed upon differentiation with the cytokine mix originating from *B. vulgatus*- and *E. coli*- stimulated BMDCs. Cytokines such as IL-6 serve as T cell survival factors and are secreted in higher amounts by BMDCs upon contact with bacterial antigens (**Figures 2C** and **3C**) (64). IL-10 is known to exert a critical role in limiting immune-mediated inflammation and to prevent autoimmune pathologies. IL-10 is broadly expressed by many cell types of the innate and adaptive immune system, serving as feedback negative regulator of the innate effector functions of macrophages, DCs and, indirectly, T cells. Furthermore, IL-10 stimulates its own production by enhancing differentiation of IL-10-secreting Tregs (65). Interestingly, creating an "imbalanced" pro-inflammatory cytokine environment by neutralizing IL-10 in the cytokine mixes led to a slightly decreased survival in all conditions tested. Nevertheless, it also increased differentiation of effector T cells. The cytokine mix secreted by *E. coli*–stimulated BMDCs induced significantly higher amounts of Th2 cells upon neutralization of IL-10, suggesting a Th2-inhibiting action of IL-10 upon exposure to strong stimuli. Since overshooting Th2 responses provoke allergic reactions, a strategy for inducing IL-10-secreting DCs with strong stimuli such as bacteria or bacterial components is of large therapeutic interest (66). Furthermore, DC-secreted IL-10 also appears to inhibit Th1 differentiation upon a strong stimulation, here represented by *E. coli*-stimulation. This effect could be abolished by neutralization of IL-10 and was less evident with the other cytokine mixes used. Neutralization of IL-10 in *B. vulgatus*-induced cytokine mixes resulted in significantly increased differentiation of Th17 cells, especially of those expressing Foxp3 and IFNγ. However, the percentage of induced $CD4^+$ T cells was relatively low, questioning the biological relevance of the observed differences. Comparatively high amounts of induced $Foxp3^+$ Tregs could be observed by cytokine mixes produced by unstimulated immature BMDCs, which was not significantly influenced by IL-10-neutralization. Immature DCs are known to promote T cell anergy and generate Tregs (5). Here, we suggest a Treg-promoting effect by immature DCs independent of antigen presentation and IL-10, which needs further investigation.

When we evaluated the immuno-modulating effects of *B. vulgatus* and *E. coli* under inflammatory conditions in a genetically predisposed host with a presumably dysbiotic microbiota, *E. coli* administration induced colitis slightly but not significantly quicker than an unchanged microbiota or *B. vulgatus*administration as indicated by accelerated weight loss beginning 3 weeks after T cell transfer (**Figure 5**). Nonetheless, flow cytometry analysis of the cLP immune cells revealed significant differences in $CD4^+$ T cell subsets even though the absolute numbers of $CD4^+$ T cells remained equal: In *B. vulgatus*-administered T cell-transplanted *Rag1*$^{-/-}$ mice, numbers of regulatory and anti-inflammatory T helper subsets were higher than upon *E. coli* administration or in T cell-transplanted control mice, indicating a potent regulation of inflammation (**Figure 6**). In contrast, *E. coli* administration resulted in high numbers of pro-inflammatory Th1 and Th1-like Th17 cells in the cLP, indicating an uncontrolled inflammation, which was not dampened by low numbers of regulatory $CD4^+$ T cell phenotypes. Administration of commensals thus seems to manipulate the Th1/Th2/Th17/Treg balance as well as the pathogenicity of induced Th17 cells. In previous studies, we had already observed that *B. vulgatus* impaired inflammation in T cell-transplanted *Rag1*$^{-/-}$ mice, whereas transplantation of *Enterobacteriaceae*-rich microbiota strongly exacerbated the course of colitis (27, 33). So far, the cellular mechanisms underlying the protective effect of *B. vulgatus* remained unknown. Here, we could shed light on the influence of the two commensal bacteria on T cell polarization and disease progression.

In addition to the *in vitro* experiments, we observed increased *Nfkbiz* gene expression in inflamed colonic tissue isolated from T cell-transplanted *Rag1*$^{-/-}$ mice administered with *E. coli*-compared to control or *B. vulgatus*-administered mice. This finding could not be completely traced back to intestinal DCs as sole source of IκBζ-expressing cells. Since mouse small intestinal epithelial cells also increased IκBζ expression upon stimulation with *E. coli in vitro* (**Figures 2D, E**), we assume that the measured *Nfkbiz* expression originated from intestinal epithelial cells.We are aware of the limitation that small intestinal cells do not fully recapitulate the response of colonic tissue and might not be a colonization site for these commensals.

In IBD, the colonic barrier is weakened, resulting in a close contactof the commensals with the epithelial layer (67). This increased contact to epithelial cells represents an antigen-overload, and can lead to an inappropriate and dysregulated response of $CD4^+$ T cells, resulting in pro-inflammatory phenotypes and, eventually, in chronic inflammation.

We cannot rule out a contribution of DC-derived IκBζ to the consolidation of inflammation. Intestinal DCs migrate to the mLN upon antigenic challengewhere they present microbiota-derived antigens to naïve T cells thus initiating an adaptive immune response. Recognition of the cognate antigen along with DC-secreted lineage specifying cytokines leads to the differentiation and proliferation of effector T cells, which migrate to the effector

site, e.g., the cLP (68, 69). On a first sight, low IκBζ levels observed *in vivo* in cLP and mLN DCs in bacteria-administered T cell-transplanted *Rag1*$^{-/-}$ mice would contradict the *in vitro* findings. Ir is however conceivable that cLP and mLN DCs represent DCs in different stages of maturation and differentiation. An earlier accumulation of IκBζ results in suppression of NFκB-induced gene transcription due to the inhibitory activity of IκBζ, creating a self-limiting negative feedback loop. We speculate that the *in vivo*-induced IκBζ expression levels are already diminished in the analyzed cLP and mLN DCs as a result of its self-limitation at a later time point of the maturation stage. WT BMDCs stimulated with heat-inactivated microbiota samples of T cell-transplanted *Rag1*$^{-/-}$ mice from the experiment discussed above confirmed the commensal-dependent IκBζ expression in DCs: Supporting the inflammation-dampening influence of *B. vulgatus* is the significantly decreased secretion of IL-23 by BMDCs stimulated with the microbiota of *B. vulgatus*-administered mice, a cytokine responsible not only for the maintenance of Th17 cells but also for the innate immune-based pathology (70).

In conclusion, our study suggests that modulating the host's immune response by commensal bacteria can define the outcome of a Th17-mediated disease, at least in part, *via* regulation of IκBζ in DCs. These findings can be applied for the optimization of microbiota-based therapeutic strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the Basel Declaration. Protocols and experiments involving mice were reviewed and approved by the responsible Institutional Review Committee and the local authorities within H5/10, H1/15, §4 09.01.2015, §4 14.06.2017 and §4 28.09.2017 approval.

AUTHOR CONTRIBUTIONS

LM, AlS, KS-O, and J-SF conceived and designed the experiments. LM, MT, H-CL, JK, CK, AL, AG, AnS, and SM performed the experiments. LM, MT, HC-L, and J-SF analyzed the data. LM, AL, and J-SF wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy—EXC-2124 and Collaborative Research Centres 685 (CRC685), the DFG research training group 1708, the Bundesministerium für Bildung und Forschung (BMBF), and the German Center for Infection Research (DZIF).

ACKNOWLEDGMENTS

We would like to thank Dr. Libera Lo Presti for her support in editing and proof-reading the manuscript. We acknowledge support by Open Access Publishing Fund of University of Tübingen.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.612336/full#supplementary-material

REFERENCES

1. Rutella S, Locatelli F. Intestinal dendritic cells in the pathogenesis of inflammatory bowel disease. *World J Gastroenterol* (2011) 17(33):3761–75. doi: 10.3748/wjg.v17.i33.3761
2. Zhao Q, Elson CO. Adaptive immune education by gut microbiota antigens. *Immunology* (2018) 154(1):28–37. doi: 10.1111/imm.12896
3. Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. *Nat Rev Immunol* (2017) 17(4):219–32. doi: 10.1038/nri.2017.7
4. Sohail MU, Althani A, Anwar H, Rizzi R, Marei HE. Role of the Gastrointestinal Tract Microbiome in the Pathophysiology of Diabetes Mellitus. *J Diabetes Res* (2017) 2017:9631435. doi: 10.1155/2017/9631435
5. Steimle A, Frick JS. Molecular Mechanisms of Induction of Tolerant and Tolerogenic Intestinal Dendritic Cells in Mice. *J Immunol Res* (2016) 2016:1958650. doi: 10.1155/2016/1958650
6. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. *Front Immunol* (2014) 5:461. doi: 10.3389/fimmu.2014.00461
7. Granucci F, Ricciardi-Castagnoli P. Interactions of bacterial pathogens with dendritic cells during invasion of mucosal surfaces. *Curr Opin Microbiol* (2003) 6(1):72–6. doi: 10.1016/S1369-5274(03)00007-9
8. Frick JS, Zahir N, Muller M, Kahl F, Bechtold O, Lutz MB, et al. Colitogenic and non-colitogenic commensal bacteria differentially trigger DC maturation and Th cell polarization: an important role for IL-6. *Eur J Immunol* (2006) 36 (6):1537–47. doi: 10.1002/eji.200635840
9. Frick JS, Grunebach F, Autenrieth IB. Immunomodulation by semi-mature dendritic cells: a novel role of Toll-like receptors and interleukin-6. *Int J Med Microbiol* (2010) 300(1):19–24. doi: 10.1016/j.ijmm.2009.08.010
10. Fu SH, Chien MW, Hsu CY, Liu YW, Sytwu HK. Interplay between Cytokine Circuitry and Transcriptional Regulation Shaping Helper T Cell Pathogenicity and Plasticity in Inflammatory Bowel Disease. *Int J Mol Sci* (2020) 21(9):3379. doi: 10.3390/ijms21093379
11. Lee GR. The Balance of Th17 versus Treg Cells in Autoimmunity. *Int J Mol Sci* (2018) 19(3):730. doi: 10.3390/ijms19030730
12. Stockinger B, Omenetti S. The dichotomous nature of T helper 17 cells. *Nat Rev Immunol* (2017) 17(9):535–44. doi: 10.1038/nri.2017.50

13. Monin L, Gaffen SL. Interleukin 17 Family Cytokines: Signaling Mechanisms, Biological Activities, and Therapeutic Implications. *Cold Spring Harb Perspect Biol* (2018) 10(4):a028522. doi: 10.1101/cshperspect.a028522
14. Agalioti T, Villablanca EJ, Huber S, Gagliani N. TH17cell plasticity: The role of dendritic cells and molecular mechanisms. *J Autoimmun* (2018) 87:50–60. doi: 10.1016/j.jaut.2017.12.003
15. Zenobia C, Hajishengallis G. Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontol 2000* (2015) 69(1):142–59. doi: 10.1111/prd.12083
16. Okamoto K, Iwai Y, Oh-Hora M, Yamamoto M, Morio T, Aoki K, et al. IkappaBzeta regulates T(H)17 development by cooperating with ROR nuclear receptors. *Nature* (2010) 464(7293):1381–5. doi: 10.1038/nature08922
17. Yamazaki S, Muta T, Takeshige K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J Biol Chem* (2001) 276(29):27657–62. doi: 10.1074/jbc.M103426200
18. Hildebrand DG, Alexander E, Horber S, Lehle S, Obermayer K, Munck NA, et al. IkappaBzeta is a transcriptional key regulator of CCL2/MCP-1. *J Immunol* (2013) 190(9):4812–20. doi: 10.4049/jimmunol.1300089
19. Amatya N, Childs EE, Cruz JA, Aggor FEY, Garg AV, Berman AJ, et al. IL-17 integrates multiple self-reinforcing, feed-forward mechanisms through the RNA binding protein Arid5a. *Sci Signal* (2018) 11(551):eaat4617. doi: 10.1126/scisignal.aat4617
20. Sundaram K, Rahman MA, Mitra S, Knoell DL, Woodiga SA, King SJ, et al. IkappaBzeta Regulates Human Monocyte Pro-Inflammatory Responses Induced by Streptococcus pneumoniae. *PLoS One* (2016) 11(9):e0161931. doi: 10.1371/journal.pone.0161931
21. Yamamoto M, Yamazaki S, Uematsu S, Sato S, Hemmi H, Hoshino K, et al. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature* (2004) 430(6996):218–22. doi: 10.1038/nature02738
22. Belikan P, Buhler U, Wolf C, Pramanik GK, Gollan R, Zipp F, et al. CCR7 on CD4(+) T Cells Plays a Crucial Role in the Induction of Experimental Autoimmune Encephalomyelitis. *J Immunol* (2018) 200(8):2554–62. doi: 10.4049/jimmunol.1701419
23. Waidmann M, Bechtold O, Frick JS, Lehr HA, Schubert S, Dobrindt U, et al. Bacteroides vulgatus protects against Escherichia coli-induced colitis in gnotobiotic interleukin-2-deficient mice. *Gastroenterology* (2003) 125 (1):162–77. doi: 10.1016/S0016-5085(03)00672-3
24. Shiina T, Konno A, Oonuma T, Kitamura H, Imaoka K, Takeda N, et al. Targeted disruption of MAIL, a nuclear IkappaB protein, leads to severe atopic dermatitis-like disease. *J Biol Chem* (2004) 279(53):55493–8. doi: 10.1074/jbc.M409770200
25. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* (1999) 223 (1):77–92. doi: 10.1016/S0022-1759(98)00204-X
26. Steimle A, Michaelis L, Di Lorenzo F, Kliem T, Munzner T, Maerz JK, et al. Weak Agonistic LPS Restores Intestinal Immune Homeostasis. *Mol Ther* (2019) 27(11):1974–91. doi: 10.1016/j.ymthe.2019.07.007
27. Gronbach K, Flade I, Holst O, Lindner B, Ruscheweyh HJ, Wittmann A, et al. Endotoxicity of lipopolysaccharide as a determinant of T-cell-mediated colitis induction in mice. *Gastroenterology* (2014) 146(3):765–75. doi: 10.1053/j.gastro.2013.11.033
28. Di Lorenzo F, Pither MD, Martufi M, Scarinci I, Guzman-Caldentey J, Lakomiec E, et al. Pairing Bacteroides vulgatus LPS Structure with Its Immunomodulatory Effects on Human Cellular Models. *ACS Cent Sci* (2020) 6(9):1602–16. doi: 10.1021/acscentsci.0c00791
29. Lange A, Schafer A, Bender A, Steimle A, Beier S, Parusel R, et al. Galleria mellonella: A Novel Invertebrate Model to Distinguish Intestinal Symbionts From Pathobionts. *Front Immunol* (2018) 9:2114. doi: 10.3389/fimmu.2018.02114
30. Krajina T, Leithauser F, Reimann J. MHC class II-independent CD25+ CD4+ CD8alpha beta+ alpha beta T cells attenuate CD4+ T cell-induced transfer colitis. *Eur J Immunol* (2004) 34(3):705–14. doi: 10.1002/eji.200324463
31. Hermann-Bank ML, Skovgaard K, Stockmarr A, Larsen N, Molbak L. The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity. *BMC Genomics* (2013) 14:788. doi: 10.1186/1471-2164-14-788
32. Sato K, Kumita W, Ode T, Ichinose S, Ando A, Fujiyama Y, et al. OmpA variants affecting the adherence of ulcerative colitis-derived Bacteroides vulgatus. *J Med Dent Sci* (2010) 57(1):55–64. doi: 10.11480/jmds.570107
33. Steimle A, Gronbach K, Beifuss B, Schafer A, Harmening R, Bender A, et al. Symbiotic gut commensal bacteria act as host cathepsin S activity regulators. *J Autoimmun* (2016) 75:82–95. doi: 10.1016/j.jaut.2016.07.009
34. Maerz JK, Trostel C, Lange A, Parusel R, Michaelis L, Schafer A, et al. Bacterial Immunogenicity Is Critical for the Induction of Regulatory B Cells in Suppressing Inflammatory Immune Responses. *Front Immunol* (2019) 10:3093. doi: 10.3389/fimmu.2019.03093
35. Kempski J, Brockmann L, Gagliani N, Huber S. TH17 Cell and Epithelial Cell Crosstalk during Inflammatory Bowel Disease and Carcinogenesis. *Front Immunol* (2017) 8:1373. doi: 10.3389/fimmu.2017.01373
36. Martinez-Sanchez ME, Huerta L, Alvarez-Buylla ER, Villarreal Lujan C. Role of Cytokine Combinations on CD4+ T Cell Differentiation, Partial Polarization, and Plasticity: Continuous Network Modeling Approach. *Front Physiol* (2018) 9:877. doi: 10.3389/fphys.2018.00877
37. Brucklacher-Waldert V, Ferreira C, Innocentin S, Kamdar S, Withers DR, Kullberg MC, et al. Tbet or Continued RORgammat Expression Is Not Required for Th17-Associated Immunopathology. *J Immunol* (2016) 196 (12):4893–904. doi: 10.4049/jimmunol.1600137
38. Moudgil KD, Choubey D. Cytokines in autoimmunity: role in induction, regulation, and treatment. *J Interferon Cytokine Res* (2011) 31(10):695–703. doi: 10.1089/jir.2011.0065
39. Gartlan KH, Varelias A, Koyama M, Robb RJ, Markey KA, Chang K, et al. Th17 plasticity and transition toward a pathogenic cytokine signature are regulated by cyclosporine after allogeneic SCT. *Blood Adv* (2017) 1(6):341–51. doi: 10.1182/bloodadvances.2016002980
40. Hovhannisyan Z, Treatman J, Littman DR, Mayer L. Characterization of interleukin-17-producing regulatory T cells in inflamed intestinal mucosa from patients with inflammatory bowel diseases. *Gastroenterology* (2011) 140 (3):957–65. doi: 10.1053/j.gastro.2010.12.002
41. Wu X, Tian J, Wang S. Insight Into Non-Pathogenic Th17 Cells in Autoimmune Diseases. *Front Immunol* (2018) 9:1112. doi: 10.3389/fimmu.2018.01112
42. Leppkes M, Becker C, Ivanov II, Hirth S, Wirtz S, Neufert C, et al. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* (2009) 136(1):257–67. doi: 10.1053/j.gastro.2008.10.018
43. Reinoso Webb C, den Bakker H, Koboziev I, Jones-Hall Y, Rao Kottapalli K, Ostanin D, et al. Differential Susceptibility to T Cell-Induced Colitis in Mice: Role of the Intestinal Microbiota. *Inflamm Bowel Dis* (2018) 24(2):361–79. doi: 10.1093/ibd/izx014
44. Kiesler P, Fuss IJ, Strober W. Experimental Models of Inflammatory Bowel Diseases. *Cell Mol Gastroenterol Hepatol* (2015) 1(2):154–70. doi: 10.1016/j.jcmgh.2015.01.006
45. Hirahara K, Nakayama T. CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. *Int Immunol* (2016) 28(4):163–71. doi: 10.1093/intimm/dxw006
46. Kamada N, Seo SU, Chen GY, Nunez G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* (2013) 13(5):321–35. doi: 10.1038/nri3430
47. Johnson EL, Heaver SL, Walters WA, Ley RE. Microbiome and metabolic disease: revisiting the bacterial phylum Bacteroidetes. *J Mol Med (Berl)* (2017) 95(1):1–8. doi: 10.1007/s00109-016-1492-2
48. DeGruttola AK, Low D, Mizoguchi A, Mizoguchi E. Current Understanding of Dysbiosis in Disease in Human and Animal Models. *Inflamm Bowel Dis* (2016) 22(5):1137–50. doi: 10.1097/MIB.0000000000000750
49. Chen YL, Chen YT, Lo CF, Hsieh CI, Chiu SY, Wu CY, et al. Early Detection of T cell Transfer-induced Autoimmune Colitis by In Vivo Imaging System. *Sci Rep* (2016) 6:35635. doi: 10.1038/srep35635
50. Lorscheid S, Muller A, Loffler J, Resch C, Bucher P, Kurschus FC, et al. Keratinocyte-derived IkappaBzeta drives psoriasis and associated systemic inflammation. *JCI Insight* (2019) 4(22):e130835. doi: 10.1172/jci.insight.130835
51. Muller A, Hennig A, Lorscheid S, Grondona P, Schulze-Osthoff K, Hailfinger S, et al. IkappaBzeta is a key transcriptional regulator of IL-36-driven

psoriasis-related gene expression in keratinocytes. *Proc Natl Acad Sci U S A* (2018) 115(40):10088–93. doi: 10.1073/pnas.1801377115
52. Okuma A, Hoshino K, Ohba T, Fukushi S, Aiba S, Akira S, et al. Enhanced apoptosis by disruption of the STAT3-IkappaB-zeta signaling pathway in epithelial cells induces Sjogren's syndrome-like autoimmune disease. *Immunity* (2013) 38(3):450–60. doi: 10.1016/j.immuni.2012.11.016
53. May MJ, Ghosh S. Signal transduction through NF-kappa B. *Immunol Today* (1998) 19(2):80–8. doi: 10.1016/S0167-5699(97)01197-3
54. McFarland BC, Hong SW, Rajbhandari R, Twitty GBJr., Gray GK, Yu H, et al. NF-kappaB-induced IL-6 ensures STAT3 activation and tumor aggressiveness in glioblastoma. *PLoS One* (2013) 8(11):e78728. doi: 10.1371/journal.pone.0078728
55. Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* (2017) 2:e17023. doi: 10.1038/sigtrans.2017.23
56. Cao S, Zhang X, Edwards JP, Mosser DM. NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. *J Biol Chem* (2006) 281(36):26041–50. doi: 10.1074/jbc.M602222200
57. Nanki K, Fujii M, Shimokawa M, Matano M, Nishikori S, Date S, et al. Somatic inflammatory gene mutations in human ulcerative colitis epithelium. *Nature* (2020) 577(7789):254–9. doi: 10.1038/s41586-019-1844-5
58. Muller M, Fink K, Geisel J, Kahl F, Jilge B, Reimann J, et al. Intestinal colonization of IL-2 deficient mice with non-colitogenic B. vulgatus prevents DC maturation and T-cell polarization. *PLoS One* (2008) 3(6):e2376. doi: 10.1371/journal.pone.0002376
59. Sato S, Nomura F, Kawai T, Takeuchi O, Muhlradt PF, Takeda K, et al. Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J Immunol* (2000) 165(12):7096–101. doi: 10.4049/jimmunol.165.12.7096
60. Horber S, Hildebrand DG, Lieb WS, Lorscheid S, Hailfinger S, Schulze-Osthoff K, et al. The Atypical Inhibitor of NF-kappaB, IkappaBzeta, Controls Macrophage Interleukin-10 Expression. *J Biol Chem* (2016) 291(24):12851–61. doi: 10.1074/jbc.M116.718825
61. Willems M, Dubois N, Musumeci L, Bours V, Robe PA. IkappaBzeta: an emerging player in cancer. *Oncotarget* (2016) 7(40):66310–22. doi: 10.18632/oncotarget.11624
62. Hiscott J, Marois J, Garoufalis J, D'Addario M, Roulston A, Kwan I, et al. Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Mol Cell Biol* (1993) 13(10):6231–40. doi: 10.1128/MCB.13.10.6231
63. Kim J, Ahn H, Yu S, Ahn JH, Ko HJ, Kweon MN, et al. IkappaBzeta controls NLRP3 inflammasome activation via upregulation of the Nlrp3 gene. *Cytokine* (2020) 127:154983. doi: 10.1016/j.cyto.2019.154983
64. Li B, Jones LL, Geiger TL. IL-6 Promotes T Cell Proliferation and Expansion under Inflammatory Conditions in Association with Low-Level RORgammat Expression. *J Immunol* (2018) 201(10):2934–46. doi: 10.4049/jimmunol.1800016
65. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* (2010) 10(3):170–81. doi: 10.1038/nri2711
66. Schulke S. Induction of Interleukin-10 Producing Dendritic Cells As a Tool to Suppress Allergen-Specific T Helper 2 Responses. *Front Immunol* (2018) 9:455. doi: 10.3389/fimmu.2018.00455
67. Antoni L, Nuding S, Wehkamp J, Stange EF. Intestinal barrier in inflammatory bowel disease. *World J Gastroenterol* (2014) 20(5):1165–79. doi: 10.3748/wjg.v20.i5.1165
68. Chewning JH, Weaver CT. Development and survival of Th17 cells within the intestines: the influence of microbiome- and diet-derived signals. *J Immunol* (2014) 193(10):4769–77. doi: 10.4049/jimmunol.1401835
69. De Calisto J, Villablanca EJ, Wang S, Bono MR, Rosemblatt M, Mora JR. T-cell homing to the gut mucosa: general concepts and methodological considerations. *Methods Mol Biol* (2012) 757:411–34. doi: 10.1007/978-1-61779-166-6_24
70. Hue S, Ahern P, Buonocore S, Kullberg MC, Cua DJ, McKenzie BS, et al. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* (2006) 203(11):2473–83. doi: 10.1084/jem.20061099

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 20 January 2021
doi: 10.3389/fimmu.2020.599623

Vitamin D3-Induced Tolerogenic Dendritic Cells Modulate the Transcriptomic Profile of T CD4$^+$ Cells Towards a Functional Hyporesponsiveness

*Juan Navarro-Barriuso [1,2], María José Mansilla [1,2], Bibiana Quirant-Sánchez [1,2], Aina Teniente-Serra [1,2], Cristina Ramo-Tello [3] and Eva M. Martínez-Cáceres [1,2]**

[1] Division of Immunology, LCMN, Germans Trias i Pujol University Hospital and Research Institute, Barcelona, Spain, [2] Department of Cellular Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain, [3] Multiple Sclerosis Unit, Department of Neurosciences, Germans Trias i Pujol University Hospital, Barcelona, Spain

The use of autologous tolerogenic dendritic cells (tolDC) has become a promising alternative for the treatment of autoimmune diseases. Among the different strategies available, the use of vitamin D3 for the generation of tolDC (vitD3-tolDC) constitutes one of the most robust approaches due to their immune regulatory properties, which are currently being tested in clinical trials. However, the mechanisms that vitD3-tolDC trigger for the induction of tolerance remain elusive. For this reason, we performed a full phenotypical, functional, and transcriptomic characterization of T cells upon their interaction with autologous, antigen-specific vitD3-tolDC. We observed a strong antigen-specific reduction of T cell proliferation, combined with a decrease in the relative prevalence of T_H1 subpopulations and IFN-γ production. The analysis of the transcriptomic profile of T CD4$^+$ cells evidenced a significant down-modulation of genes involved in cell cycle and cell response to mainly pro-inflammatory immune-related stimuli, highlighting the role of *JUNB* gene as a potential biomarker of these processes. Consequently, our results show the induction of a strong antigen-specific hyporesponsiveness combined with a reduction on the T_H1 immune profile of T cells upon their interaction with vitD3-tolDC, which manifests the regulatory properties of these cells and, therefore, their therapeutic potential in the clinic.

Keywords: tolerogenic dendritic cells, immune tolerance, T cells, antigen-specific response, transcriptomic study

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Ali Akbar Pourfathollah,
Tarbiat Modares University, Iran
Paulina A. García-González,
University of Chile, Chile
Willem Van Eden,
Utrecht University, Netherlands
Carolina Llanos,
Pontificia Universidad Católica de Chile, Chile
Grant P. Parnell,
Westmead Institute for Medical Research, Australia

***Correspondence:**
Eva M. Martínez-Cáceres
emmartinez.germanstrias@gencat.cat

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *27 August 2020*
Accepted: *30 November 2020*
Published: *20 January 2021*

Citation:
Navarro-Barriuso J, Mansilla MJ, Quirant-Sánchez B, Teniente-Serra A, Ramo-Tello C and Martínez-Cáceres EM (2021) Vitamin D3-Induced Tolerogenic Dendritic Cells Modulate the Transcriptomic Profile of T CD4$^+$ Cells Towards a Functional Hyporesponsiveness. Front. Immunol. 11:599623. doi: 10.3389/fimmu.2020.599623

INTRODUCTION

In the last years, tolerogenic dendritic cells (tolDC) have become one of the most promising alternatives for the treatment of autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis, or type 1 diabetes. In fact, several Phase I clinical trials have already finished or are currently ongoing, with positive results regarding the safety and the tolerability of this therapeutic cell-based approach (1). In general, tolDC are commonly defined as a stable and semi-mature subset of dendritic cells (DC),

between antigen-capturing immature DC (iDC) and immunogenic mature DC (mDC)—characterized by their increased expression of MHC class II and co-stimulatory molecules. But most importantly, tolDC are presumably capable to induce immune tolerance towards the peptides these cells are presenting, in an antigen-specific manner (2–5).

TolDC can be generated *in vitro* from peripheral blood monocytes. In the last years, a wide variety of protocols for their production have been reported, ranging from the use of different drugs and chemical agents to genetic engineering techniques (6, 7). In this regard, the use of 1,25-dyhydroxyvitamin D3, the active form of vitamin D3, constitutes one of the most widely studied approaches for the differentiation of tolDC. Briefly, vitamin D3-induced tolDC (vitD3-tolDC) are thought to develop their regulatory properties through a semi-mature profile, their ability to inhibit or reduce T cell responses, and a switch of the immune response towards a T_H2 profile (8–18). Furthermore, vitD3-tolDC are characterized by a reduced NF-κB-mediated activity and an increase of mTOR-mediated glucose metabolism (10, 19).

Even though tolDC—and vitD3-tolDC in particular—have been characterized with a developing knowledge over their metabolism, molecular mechanisms, and functional pathways, the specific effect of these cells over the rest of the immune-related components still remains elusive. It is known that tolDC can usually induce either anergy, hyporesponsiveness or depletion over activated T cells, as well as regulatory T cell (Treg) differentiation (20). However, to our knowledge, so far only one study has focused its attention on the actual processes that autologous T cells might be undergoing upon tolDC interaction —reporting an induction of hyporesponsiveness of $CD4^+$ memory and naïve T cells towards antigen-specific stimulation mediated by dexamethasone-induced tolDC (21)—but neither at the transcriptomic level nor with vitD3-tolDC in particular.

In previous studies, our group has already extensively characterized vitD3-tolDC phenotypically, functionally, and transcriptomically, evidencing the regulatory potential of these cells both *in vitro* and *in vivo* in the animal model of MS, experimental autoimmune encephalomyelitis (EAE) (13, 16, 22–24). Consequently, we wanted to take one step further for the elucidation of the mechanisms of immune tolerance induction of vitD3-tolDC. With that aim, here we present a full phenotypical, functional, and transcriptomic characterization of T $CD4^+$ cells after their interaction with autologous vitD3-tolDC loaded with tetanus toxin (TT), in order to study the antigen-specific effect mediated by these cells compared to TT-loaded immunogenic mDC. The purpose of this study is to identify one or several potential biomarkers of the immune modulation developed by vitD3-tolDC over T cells, which could constitute an interesting tool for the monitoring of patients treated with these cells in clinical trials, and the understanding of the mechanisms of tolerance induction.

MATERIAL AND METHODS

Sample Collection

Buffy coat samples from 16 randomized healthy controls were obtained from the *Banc de Sang i Teixits* (Barcelona, Spain), according to the institutional Standard Operating Procedures for blood donation, including a signed informed consent. In parallel, whole blood samples from 12 different healthy donors were collected by standard venipuncture in lithium heparin tubes for the allogeneic functional assays (see below).

Monocyte Isolation

Healthy donor buffy coat samples were processed first depleting $CD3^+$ cells using the RoseetteSep® Human Monocyte Enrichment Kit (StemCell Technologies, Vancouver, Canada) prior to a density gradient separation using ficoll-hypaque (Rafer, Zaragoza, Spain). Afterwards, $CD14^+$ cells were isolated using the EasySep® Human CD14 Positive Selection Kit (StemCell), according to the manufacturer's instructions. Cell viability was determined using 7-amino-actinomycin D (7-AAD) (BD Biosciences, Franklin Lakes, NK, USA) and phycoerythrin (PE)-conjugated annexin V (Immunotools, Friesoythe, Germany) staining for 20 min at 4°C, protected from light, and cell counts were quantified simultaneously using PerfectCount beads (Cytognos, Salamanca, Spain). Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences), and monocyte purity was determined using forward and side scatter gating strategies on FACSDiva software (BD Biosciences).

TT-Loaded DC Cultures

The protocol for the generation of antigen-loaded tolDC was adapted from a previous study (25). Briefly, isolated monocytes were cultured for 6 days in 24-well plates at 37°C at a density of 1×10^6 cells/ml in X-VIVO 15 medium, in the presence of 400 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 500 U/ml IL-4 (both from Peprotech, London, UK). The whole volume of medium and cytokines was replenished on day 4. If no further treatment was performed, monocytes were differentiated into iDC. For the generation of mDC, we further added a maturation cocktail, containing 1,000 U/ml IL-1β, 1,000 U/ml TNF-α (both from Peprotech) and 1 μM prostaglandin E2 (PGE2) (Pfizer, New York, NY, USA) on day 4. Finally, in addition to the maturation cocktail, we added 1 nM vitamin D3 (Calcijex, Abbott, Chicago, IL, USA) on days 0 and 4 for the differentiation of vitD3-tolDC. For the generation of TT-loaded mDC (mDC-TT) and TT-loaded vitD3-tolDC (vitD3-tolDC-TT) as antigen-specific experimental conditions, 0.1 μg/ml of the whole TT protein (Sigma-Aldrich, St. Louis, MO, USA) were added to the mDC and vitD3-tolDC cultures on day 3, 18 h before the addition of the maturation stimulus, while still in an immature status. On day 6, cells were harvested after an accutase (Invitrogen, Carlsbad, CA, USA) detaching treatment for 30 min, and washed twice. As shown above, cell counts and viability were determined by flow cytometry.

Autologous PBMC Isolation, Co-Culture, and Sorting

For the isolation of autologous PBMC, 3 ml of the buffy coat samples from each healthy donor was processed using a ficoll-hypaque density gradient separation and washed twice. Afterwards, cells were counted by flow cytometry, as described above, and plated in round-bottom 96-well plates at a density of

1×10^6 cells/ml in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 U/ml penicillin (Reig Jofre, Sant Joan Despí, Spain) and 100 μg/ml streptomycin (Normon, Tres Cantos, Spain). The plates were then incubated for 6 days at 37°C in a 5% CO_2 atmosphere. Afterwards, cells were harvested, and cell counts and viability were determined by flow cytometry.

Subsequently, an antigen-specific proliferation experimental setup was performed in 96-well round-bottom plates with co-cultures of 10^5 autologous PBMC and 5,000 either mDC-TT or vitD3-tolDC-TT (1:20 ratio) in a final volume of 200 μl of supplemented RPMI medium. For each condition, 48 replicates were performed. Cells were then incubated for 5 days at 37°C in a 5% CO_2 atmosphere. Afterwards, cells were harvested and the whole volume of each cell suspension was incubated for 20 min, protected from light, with the adequate amounts of monoclonal antibodies anti-CD3 Violet 450 (V450) and anti-CD4 PerCP-Cyanine dye (Cy)5.5. Finally, cells were washed and the whole $CD3^+CD4^+$ cell subpopulation was isolated and counted using a FACSAria II cell sorter (BD Biosciences). Finally, dry pellets of mDC-TT-conditioned $CD3^+CD4^+$ cells (mDC-Tcell) and vitD3-tolDC-TT-conditioned $CD3^+CD4^+$ cells (vitD3-Tcell) were obtained by centrifugation and stored at −80°C.

Phenotype Characterization of DC and Autologous PBMC

Surface expression of CD11c, CD14, CD83, CD86 and HLA-DR in iDC, mDC, mDC-TT, vitD3-tolDC and vitD3-tolDC-TT was determined by flow cytometry. In each case, DC suspensions were incubated for 20 min, protected from light, with the appropriate amounts of monoclonal antibodies anti-: CD11c PE-Cy7, CD14 V450, CD83 allophycocyanin (APC), CD86 fluorescein isothiocyanate (FITC) and HLA-DR Violet 500 (V500) (all of them from BD Biosciences). Subsequently, at least 10,000 $CD11c^+$ events of each condition were acquired using a FACSCanto II flow cytometer and analyzed using FACSDiva software.

For the phenotypical characterization of mDC-Tcell and vitD3-Tcell, cell suspensions of these conditions were incubated for 20 min with the adequate amounts of monoclonal antibodies indicated below. Afterwards, samples were washed twice and acquired on a LSRFortessa flow cytometer, setting the stopping gate at 300,000 peripheral blood mononuclear cells. The definition of each peripheral blood mononuclear cell subpopulation was determined as specified in **Supplementary Table 1**, using several combinations of the following monoclonal antibodies anti-: CXCR3 AlexaFluor (AF)488, CD4 PerCP-Cy5.5, CCR7 PE, CD45RA PE-Cy7, CD38 APC, CD45 AF700, CD8 APC-H7, CD3 V450, HLA-DR V500, CCR6 Brilliant Violet (BV) 605, CD25 PE, CCR4 PE-Cy7, CD127 AF647, CD45RO APC-H7, CD49b FITC and LAG-3 PE (BD Biosciences). Results were analyzed with FACSDiva software (BD Biosciences). Forward and side scatter gating strategy was used in order to select the desired lymphocyte subpopulations, and their relative percentages were analyzed for each cell subset.

Allogeneic and Autologous Cell Proliferation Assays

For the determination of the reactivity of PBMC from each donor against TT, 2×10^5 PBMC were plated in 96-well round bottom plates at day 0 of each culture in supplemented RPMI medium containing 0.1 μg/ml TT. As control conditions, the same number of cells was cultured with either supplemented RPMI medium only (negative control) or 50 ng/ml phorbol 12-myristate-13-acetate (PMA) and 500 ng/ml ionomycin (positive control). Ten replicates were performed for the negative control and the condition of analysis, and six replicates for the positive control. Cells were then cultured for 5 days at 37°C in a 5% CO_2 atmosphere. Afterwards, 1 μCi [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) was added to each well, and the plate was incubated for further 18 h under the same conditions. Cells were then collected using a HARVESTER96 2M cell harvester (Tomtec Inc, Hamdem, CT, USA) and read on a 1450 MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland). Donors were considered positive for TT reactivity when the counts per minute (cpm) of at least five replicates from the condition of analysis were over the mean plus two times the standard deviation (SD) of the negative control.

For the isolation of allogeneic PBMC, whole blood samples of different healthy donors were processed by ficoll-hypaque density gradient separation. Cells were washed twice, and afterwards, their absolute number and viability was determined as shown above. Subsequently, 10^5 either allogeneic or autologous viable PBMC were co-cultured with 5,000 either iDC, mDC, mDC-TT, vitD3-tolDC or vitD3-tolDC-TT (1:20 ratio) in 96-well round bottom plates, in a total volume of 200 μl of supplemented RPMI medium. Again, as negative and positive controls, either supplemented RPMI medium or a mix of 50 ng/ml PMA and 500 ng/ml ionomycin was used, respectively. Six replicates of each condition were performed. Cells were then plated for 4 days at 37°C in a 5% CO_2 atmosphere, and afterwards, 1 μCi [^{3}H]-thymidine was added to each well, and the plates were incubated, harvested, and read as described above.

Cytokine and Soluble Protein Production

The production of granzyme B (GZMB), as well as of IL-1β, IL-6, IL-10, IFN-γ, IL-12p70 and TNF-α cytokines, was quantified in the supernatants of mDC-TT and vitD3-tolDC-TT with autologous PBMC co-cultures, using the Human Soluble Protein CBA Flex Set (BD biosciences) according to manufacturer's instructions. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences), and the results were analyzed using FACSDiva software. The production of TGF-β was determined using the Human/Mouse TGF beta 1 Uncoated ELISA kit (Invitrogen) in 100 μl of the co-culture supernatants after sample activation with HCl 1N, following the manufacturer's instructions. The optical density of each well was measured at $\lambda = 450$ nm, and the optical density at $\lambda = 570$ nm was then subtracted as background signal, using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

RNA Extraction and RNA-seq Analysis

Total RNA of autologous mDC-Tcell and vitD3-Tcell samples was isolated using the automated Maxwell 16 LEV simplyRNA Purification Kit (Promega Biotech, Madison, WI, USA), including a DNAse I digestion step, according to manufacturer's instructions. Samples were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and subsequently stored at −80°C in RNAse-free tubes. RNA integrity number (RIN) was determined in an Agilent BioAnalyzer with the RNA6000 Pico assay (Agilent Technologies, Santa Clara, CA, USA). Afterwards, the sequencing libraries were prepared using the TruSeq Stranded Total RNA Sample Preparation kit (Illumina, San Diego, CA, USA) with 200 ng of total RNA per sample as input. Paired-end sequencing (2 × 75 bp) was then performed on a HiSeq-2500 instrument (Illumina). Reads were quality trimmed and adapters removed using Trimmomatic V0.30. TopHat software v2.1.0 was used to map RNA-seq reads to the human reference genome (Ensembl release 78) (26). FeatureCounts function was used to assign reads to genomic features focusing on RNA biotypes. A matrix with summarized raw counts of reads assigned through mapping to high confidence protein coding genes only was generated ("golden" annotation label), corresponding to stable and unlikely to change transcripts from the Consensus CDS (CCDS) Project. Data exploration results from hierarchical clustering and principal component analysis (PCA) in R software were used to exclude any outliers and assess sample similarities based on global gene expression patterns, and to guide the modeling design to be used for subsequent analyses.

Differential Gene Expression Analysis

Transcriptional changes at the gene level between mDC-Tcell and vitD3-Tcell were assessed using the Bioconductor DeSeq2 package in R (27). A paired sample comparison design, factoring in inter-individual differences, was applied. The results were considered statistically significant with an adjusted p-value (padj) < 0.05. We set a 20% fold change (FC) cutoff as the threshold for relevant biological effects (|FC| > 1.2).

Gene Ontology Enrichment Analysis

Unranked lists of the significant differentially upregulated (FC > 1.2 and padj < 0.05) and downregulated genes (FC < −1.2 and padj < 0.05) were tested for enrichment in Gene Ontology (GO) functional categories using the GOrilla web tool, applying the default settings for comparison to the background list of genes found in the dataset (28). We tested for enrichment in three types of GO categories: "biological process" (GOPROCESS), "molecular function" (GOFUNCTION), and "cellular component" (GOCOMPONENT). Enrichment score (ES) was defined as ES = (b/n)/(B/N), where "N" is the total number of genes in the background list, "B" is the total number of genes in N associated with a specific GO term, "n" is the number of differentially expressed genes being tested for enrichment and "b" is the number of n intersecting with B. Enrichment p-value is computed according to the hypergeometric (HG) model. False discovery rate (FDR) q-value is the Benjamini and Hochberg multiple testing correction adjusted p-value. For the i^{th} term (ranked according to p-value), the FDR q-value is the p-value multiplied by the number of GO terms assessed and divided by i.

Statistical Analysis

All the statistical analyses were performed with either parametric or non-parametric tests depending on the normality of each compared data set, as determined by the D'Agostino & Pearson test using Prism 6.0 software (GraphPad, La Jolla, CA, USA). For multiple comparisons, either the non-parametric Friedman test with Dunn's correction or the one-way ANOVA test with Geisser–Greenhouse correction were used, and analogously, either paired t tests or Wilcoxon tests for the comparisons between two groups if they were normally distributed or not, respectively. Results were expressed as mean ± SD, unless noted otherwise, and they were considered statistically significant when $p < 0.05$.

RESULTS

Functional and Phenotypical Characteristics of TT-Loaded vitD3-tolDC

Monocytes from 16 healthy donor samples were isolated (94.4 ± 2.8% purity) with viabilities of $CD14^+$ cells above 95%. After their differentiation into DC, with or without exposition to TT, cells were harvested and their purity, viability, and phenotype were determined by flow cytometry, as shown previously (25). In all cases, purity was >90%, as determined by the percentage of $CD11c^+$ cells, with a mean viability of 94.2 ± 3.3%, which was not affected by the addition of TT (**Supplementary Table 2**). The study of the phenotype of vitD3-tolDC-TT showed significant reductions in the surface expression of CD86 (77.2 ± 8.7%) and HLA-DR (79.5 ± 7.7%) compared to mDC, but more importantly, evidenced that the exposure of DC to TT on day 3 of the culture did not have an effect *per se* over the expression of these molecules, neither in vitD3-tolDC nor in mDC, since there were no relevant differences on the percentages of reduction (**Supplementary Figure 1**). The same could be observed regarding the functionality of these cells. On the one hand, as also shown in **Supplementary Figure 1**, both vitD3-tolDC and vitD3-tolDC-TT exhibited a similar and strongly reduced induction of allogeneic proliferation compared to mDC (vitD3-tolDC: 50.6 ± 30.7, $p < 0.001$; vitD3-tolDC-TT: 49.2 ± 36.7, $p = 0.001$). On the other hand, there were no statistically significant differences in the mean induction of allogeneic proliferation induced by mDC-TT compared to mDC ($p = 0.916$). Altogether, our results evidence that vitD3-tolDC-TT show the same tolerogenic properties as vitD3-tolDC, thus demonstrating that loading these cells with TT does not affect their phenotype nor their functionality.

VitD3-tolDC-TT Induce an Antigen-Specific Response Over Autologous PBMC

In order to test the antigen-specific functionality of vitD3-tolDC-TT in an autologous setup, we assessed the baseline reactivity of

each donor against the TT itself to measure their potential to respond under these conditions. As shown in **Figure 1A**, we were able to assess the TT reactivity in all of our healthy donors, but only nine of them resulted positive, according to the criteria described in the *Material and Methods* section—when the mean proliferation of at least 5 out of 10 replicates was over the mean plus two times the SD of the control condition—and reaching statistical significance ($p < 0.05$).

Subsequently, we analyzed the capability of our cells to induce proliferation over autologous PBMC. As shown in **Figure 1B**, a significant proliferation was only induced by mDC-TT, as evidenced by the statistically significant differences observed with the remaining conditions. Specifically, reductions of a 38.4 ± 44.3% ($p = 0.020$), a 40.0 ± 21.0% ($p < 0.001$), a 56.9 ± 19.2% ($p < 0.001$) and a 37.3 ± 17.4% ($p < 0.001$) were observed in iDC, mDC, vitD3-tolDC and vitD3-tolDC-TT, respectively, compared to mDC-TT. Our results therefore evidence that autologous proliferation is only primed if an antigenic peptide is presented by an immunogenic DC condition, such as mDC-TT, confirming the antigen-specific modulation developed by our cells. Furthermore, reduced autologous proliferation mediated by vitD3-tolDC-TT was observed in all donors (**Figure 1C**).

VitD3-tolDC-TT Drive a Reduction of T_H1 $CD4^+$ Cell Subpopulations

Once determined that an antigen-specific modulation was established by TT-loaded DC, we studied which changes were being induced over the autologous T lymphocytes. Therefore, we characterized the phenotype of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells using an exhaustive multiparametric flow cytometry panel, described in previous studies (29). First, our results evidenced a reduction in the prevalence of activated T $CD4^+$ cells, determined by HLA-DR and/or CD38 staining, in vitD3-Tcell compared to mDC-Tcell (Activated $CD4^+_{\text{mDC-Tcell}}$: 23.57 ± 15.81 *vs* Activated $CD4^+_{\text{vitD3-Tcell}}$: 18.52 ± 14.16; $p = 0.002$). The same effect was observed over T $CD8^+$ cells (Activated $CD8^+_{\text{mDC-Tcell}}$: 15.94 ± 12.48 *vs* Activated $CD8^+_{\text{vitD3-Tcell}}$: 11.33 ± 9.81; $p = 0.002$). Furthermore, we found a reduction in the relative percentages of $CD4^+$ T_H1 Central Memory (CM) and Effector Memory (EM) subpopulations in vitD3-T cell (T_H1 $CM_{\text{mDC-Tcell}}$: 33.98 ± 6.44 *vs* T_H1 $CM_{\text{vitD3-Tcell}}$: 30.23 ± 7.48; $p = 0.013$; T_H1 $EM_{\text{mDC-Tcell}}$: 44.46 ± 8.72 *vs* T_H1 $EM_{\text{vitD3-Tcell}}$: 40.95 ± 8.08; $p = 0.001$). All these results are shown in **Figures 2A, B**. Thus, our data suggest that vitD3-tolDC-TT are driving an antigen-specific switch towards a more anti-inflammatory—or less T_H1-like—profile over T $CD4^+$ lymphocytes. We could not

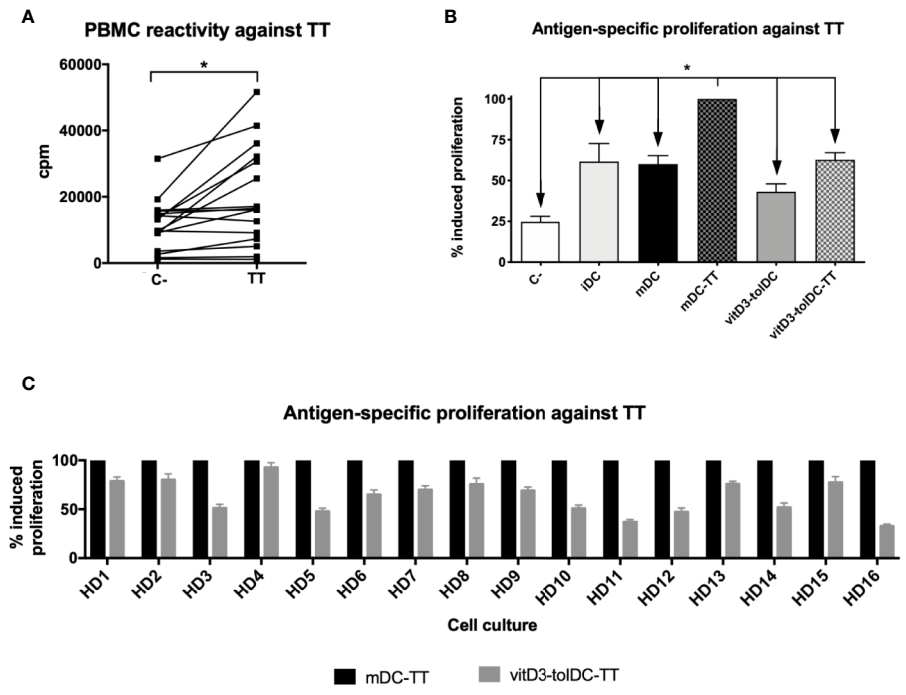


FIGURE 1 | PBMC reactivity and antigen-specific induction of autologous proliferation mediated by DC against tetanus toxin. **(A)** Induction of proliferation of PBMC without stimuli (C–) and against tetanus toxin (TT) after 5 days of culture (n = 16). Data presented as counts per minute (cpm), measured as tritiated thymidine incorporation after 18 h. Ten replicated measurements of each condition were performed. **(B)** Induction of antigen-specific autologous proliferation against TT mediated by immature DC (iDC), mature DC (mDC), TT-loaded mDC (mDC-TT), vitamin D3-induced tolerogenic DC (vitD3-tolDC) and TT-loaded vitD3-tolDC (vitD3-tolDC-TT), as well as a negative control (C–), without any stimuli (n = 16) and **(C)** comparison of autologous antigen-specific proliferation against TT mediated by mDC-TT and vitD3-tolDC-TT on each donor. Data presented as relative percentage of induced proliferation compared to mDC-TT, measured as tritiated thymidine incorporation after 18 h. Six replicated measurements of each condition were performed. Error bars corresponding to SEM. ns, not significant; *$p < 0.05$. One-way ANOVA test with Geisser–Greenhouse correction or paired t test.

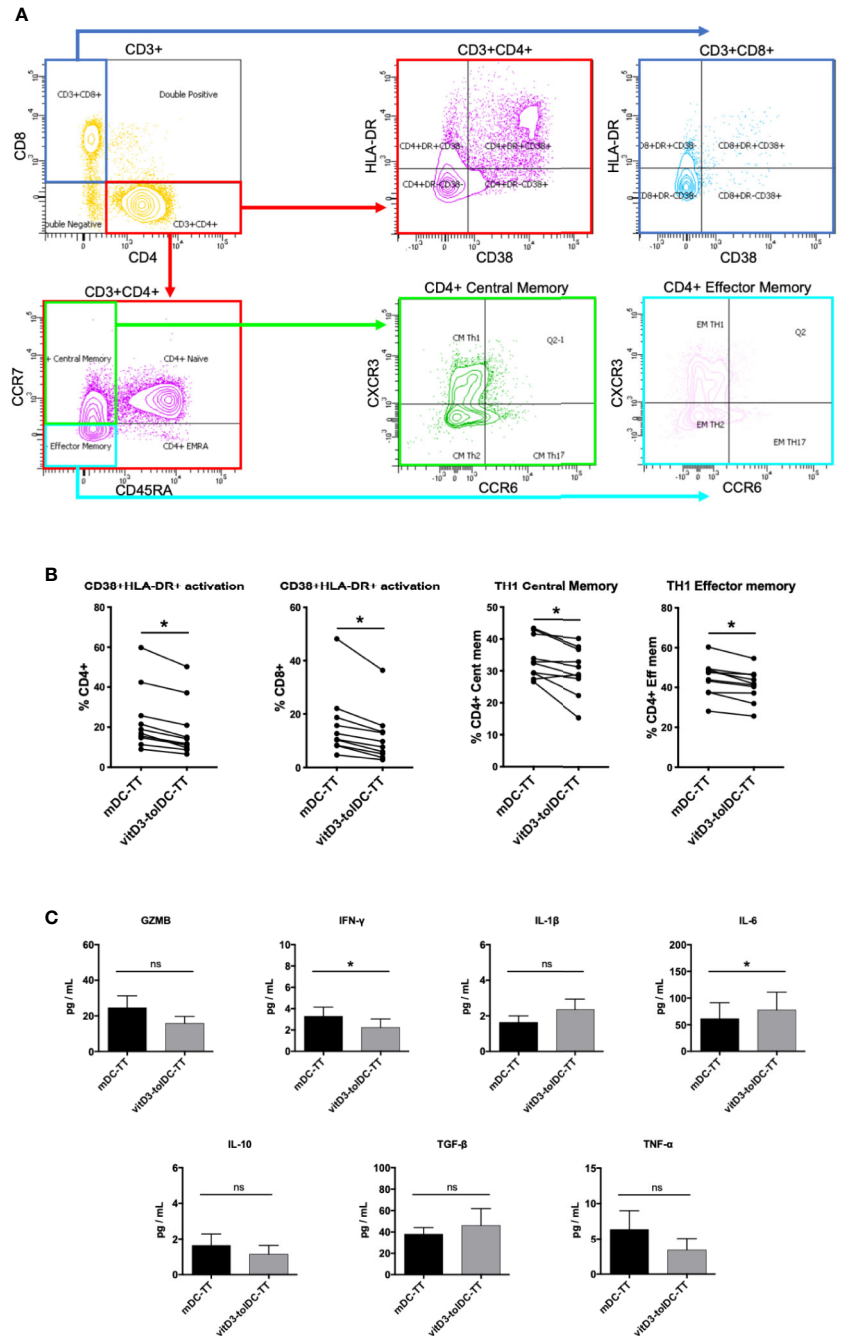


FIGURE 2 | Phenotype and cytokine production of DC-co-cultured autologous PBMC. **(A)** Gating strategy. **(B)** Relative percentages of $CD4^+CD38^+HLA\text{-}DR^+$, $CD8^+CD38^+HLA\text{-}DR^+$, T_H1 Central Memory and T_H1 Effector Memory subpopulations on PBMC co-cultured with either tetanus toxin (TT)-loaded mature DC (mDC-TT) or TT-loaded vitamin D3-induced tolerogenic DC (vitD3-tolDC-TT) after 5 days of culture (n = 10). Data presented as the relative percentage of each lymphocyte subpopulation within its respective parent subpopulation, measured by multiparametric flow cytometry, in a box and whiskers representation. Error bars corresponding to the maximum and minimum values of each condition. **(C)** Analysis of the secretion of granzyme B (GZMB) (n = 12), IFN-γ (n = 12), IL-1β (n = 8), IL-6 (n = 8), IL-10 (n = 10), TGF-β (n = 7), and TNF-α (n = 8) in the supernatants of PBMC co-cultured with either mDC-TT or vitD3-tolDC-TT after 5 days of culture using either ELISA (TGF-β) or cytometric bead array (GZMB, IFN-γ, IL-1β, IL-6, IL-10, and TNF-α) techniques. One ELISA experiment was performed for all samples, with duplicated measurements for each sample. One single cytometric bead array experiment was performed for the analysis of all the samples, with one single measurement for each sample. Sample sizes vary due to some measurements being negative or under the detection limit of the technique. Error bars corresponding to SEM. ns, not significant; * $p < 0.05$. Paired t test or Wilcoxon test.

detect any significant changes over any other T cell subpopulation, nor Treg nor Tr1 subpopulations (data not shown).

Next, we analyzed the cytokine secretion profile present in the autologous co-cultures of mDC-TT and vitD3-tolDC-TT. Our results, as shown in **Figure 2C**, evidenced a statistically significant increased secretion of the cytokine IL-6 and lower levels of IFN-γ in the co-culture of autologous PBMC with vitD3-tolDC-TT compared to mDC-TT (IL-6 $_{\text{mDC-TT}}$: 61.4 ± 84.3 pg/ml *vs* IL-6 $_{\text{vitD3-tolDC-TT}}$: 77.7 ± 94.5 pg/ml; p = 0.039; and IFN-$\gamma_{\text{mDC-TT}}$: 3.3 ± 2.9 pg/ml *vs* IFN-γ $_{\text{vitD3-tolDC-TT}}$: 2.2 ± 2.8 pg/ml; p = 0.002). Therefore, the reduction in the production of IFN-γ, combined with the increase of IL-6, again suggest a reduction of the T_H1-like cytokine profile, in line with the phenotype results. No statistically significant changes could be found in the production of GZMB, IL-1β, IL-10, TGF-β nor TNF-α.

VitD3-tolDC Induce a General Transcriptomic Repression Over T CD4$^+$ Cells

For all the 16 donors, at least 700,000 CD3$^+$CD4$^+$ cells in both conditions (mDC-Tcell and vitD3-Tcell) were successfully isolated by flow cytometry cell sorting. The gating strategy is shown in **Supplementary Figure 1**. Afterwards, we extracted their RNA and selected 10 donors that showed sufficient nucleic acid concentration and integrity for the RNA-seq analysis (RIN > 7) in both mDC-Tcell and vitD3-Tcell conditions. Consequently, donors HD4, HD5, HD9, HD10, HD11, and HD12 were discarded from downstream studies.

After processing the samples through the RNA-seq analysis, 39% of total reads could be assigned to different known RNA classes (**Supplementary Figure 2A**), and out of them, around 47% of these assigned reads could be related to protein coding genes (**Supplementary Figure 2B**). Interestingly, the hierarchical clustering analysis revealed that our samples tended to cluster by individual rather than by treatment (**Figure 3A**), but also that there is a consistent pattern by which vitD3-Tcell samples ranked higher on both axes from the PCA (**Figure 3B**). These results led to choose a paired comparative analysis approach for the differential expression analysis.

After the subsequent filtering process described in the *Material and Methods* section, a total of 16,333 protein coding genes with detectable reads were tested for differential expression. Among all of them, 546 genes showed a statistically significant change in their expression (adjusted p < 0.05) in vitD3-Tcell compared to mDC-Tcell, and only 373 also presented an absolute value of FC superior to 1.20 (|FC $_{\text{vitD3-Tcell}}$ *vs* $_{\text{mDC-Tcell}}$| > 1.20). While only 29 of these

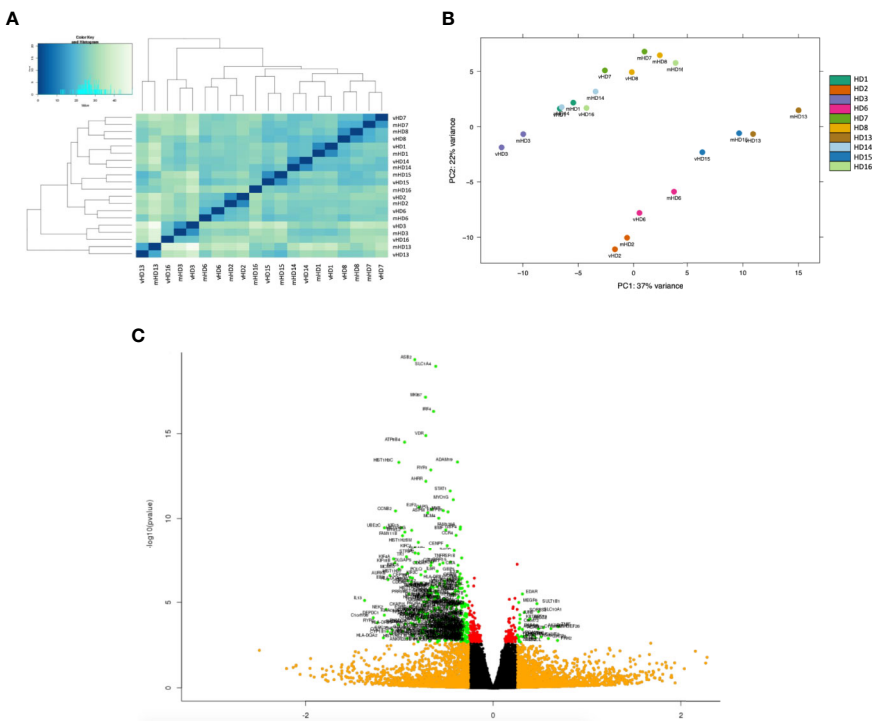


FIGURE 3 | Exploratory analysis of the RNA-seq study of T CD4+ cells co-cultured with autologous antigen-specific DC. **(A)** Hierarchical clustering analysis by gene expression of the 20 samples of CD4$^+$ T cells of the RNA-seq study. **(B)** Representation of the first two principal component analysis (PCA) components at the gene level. Each color corresponds to a different sample of CD4+ T cells, as depicted in the legend, and the co-culture condition of each sample is indicated with a prefix, either "m" for tetanus toxin (TT)-loaded mature DC or "v" for TT-loaded vitamin D3-induced tolerogenic DC. **(C)** Volcano plot showing the significant differentially expressed genes. Axis is the log2 fold change. Color code: green, significantly regulated genes (padj < 0.05; |FC| > 1.2) considered in the Gene Ontology enrichment analysis; orange, genes with |FC| > 1.2 below the significance threshold; red, genes with padj < 0.05 below the relevant fold change cutoff.

genes were up-modulated in vitD3-Tcell compared to mDC-Tcell, the majority of them, 344 genes, were down-modulated, indicating a strong transcriptomic repression induced by vitD3-tolDC-TT over these cells (**Figure 3C**).

T CD4$^+$ Cells Selectively Undergo a Strong Functional and Immune-Related Transcriptomic Down-Modulation Upon Interaction With vitD3-tolDC

When we studied those differentially expressed genes that appeared up-modulated (FC $_{\text{vitD3-Tcell } vs \text{ mDC-Tcell}} > 1.20$) in our analysis (**Table 1**), we did not find many relevant or immune-related genes. Specifically, 18 of these 29 genes did not have any GO annotation, and among the rest, we could only find the genes encoding the JUNB and SCML1 transcription factors and several other genes encoding different molecule transporters (*ABCC2* and *SLC10A1*), G-protein modulators (*GRTP1* and *RASA4*) and kinases (*AK5* and *CKMT2*).

However, as mentioned above, the study of the down-modulated genes (FC $_{\text{vitD3-Tcell } vs \text{ mDC-Tcell}} < 1.20$) yielded many more results. Within the 50 most down-regulated results (FC $_{\text{vitD3-Tcell } vs \text{ mDC-Tcell}} < 1.78$) we found several genes encoding proteins involved in the immune response (*CCL17*, *CCL22*, *EBI3*, *IL13* and *LIF*), antigen presentation (*HLA-DQA1*, *HLA-DQA2* and *HLA-DRB5*) and microtubule binding (*KIF4A*, *KIF15*, *KIF18B* and *KIFC1*), among others (**Table 2**). Furthermore, when we analyzed the whole list, we could find not only several more genes included in these categories, but also many other genes encoding proteins related to cytoskeleton and cell adhesion (*ARPC1B*, *CAPG*, *CTNNA1*, *LGALS1*, *MYL6B*, *LGALS9* or *SDC4*), actin related functionalities (*ACTB*, *ACTG1*, *PARVB* or *TPM4*), G-proteins and modulators (*GBP2*, *GBP4*, *GNA15*, *GNG4*, *IQGAP3*, *MYO1G*, *MYO1E* or *SRGAP3*), nucleic acid binding (*ASF1B*, *DEPDC1*, *EXO1* or *FEN1*), histones (*HIST1H2BL*, *HIST2H2BF* or, *HIST1H4H*), the pro-inflammatory transcription factor STAT1 and other pro-inflammatory mediators (*TNFSF4*), different kinase activators and modulators, proteases and protease inhibitors, oxydases, oxygenases, transferases and many other metabolic mediators. The whole list is shown in **Supplementary Table 3**. Altogether, these results indicate that vitD3-tolDC-TT mediate a strong down-modulation of metabolic and immune-related functions over vitD3-Tcell.

TABLE 1 | Up-modulated genes in vitD3-Tcell compared to mDC–Tcell.

Gene Symbol	GO annotation	FC vs mDC–Tcell	Adj. p-value
TMIE	NA	1.62	0.01199
PRH2	NA	1.61	0.04136
ARHGEF26	NA	1.53	0.01404
GRTP1	G-Protein Modulator; Cysteine Protease	1.50	0.03547
AKAP6	NA	1.45	0.01310
CKMT2	Amino Acid Kinase	1.43	0.03068
SLC10A1	Cation Transporter	1.40	0.00264
C17orf107	NA	1.40	0.01419
SULT1B1	NA	1.38	0.00117
TTC16	NA	1.37	0.03271
TEC	NA	1.35	0.02676
KRT72	NA	1.31	0.00543
ABCC2	ATP-Binding Cassette (ABC) Transporter	1.30	0.00598
KRT73	NA	1.28	0.02747
SORBS3	NA	1.26	0.00279
AK5	Nucleotide Kinase	1.24	0.02996
EDAR	NA	1.24	0.00041
ADAM23	Metalloprotease	1.24	0.01495
RALGPS2	Guanyl-Nucleotide Exchange Factor	1.23	0.02922
ALS2CL	NA	1.22	0.04815
KBTBD11	NA	1.22	0.00543
JUNB	Basic Leucine Zipper Transcription Factor; Nucleic Acid Binding	1.22	0.00360
RASA4	G-Protein Modulator	1.22	0.01328
C9orf72	NA	1.22	0.00818
ZC4H2	NA	1.21	0.04572
ADPRM	NA	1.21	0.01438
SCML1	Chromatin/Chromatin-Binding Protein; Transcription Factor	1.21	0.03955
MEGF6	Extracellular Matrix Protein	1.21	0.00102
LMTK3	NA	1.21	0.03156

Gene expression values from an RNA-seq analysis with healthy donors (n = 10). Data presented as the mean fold change (FC) of expression in vitD3-Tcell compared to mDC-Tcell. GO, Gene Ontology; mDC-Tcell, mature dendritic cell-conditioned CD3$^+$CD4$^+$ cells; NA, not available; padj, adjusted p-value; vitD3-Tcell, vitamin D3-induced tolerogenic dendritic cell-conditioned CD3$^+$CD4$^+$ cells.

VitD3-Tcell Present Decreased Cell Cycle and Mitotic Activity

In this regard, the GO enrichment analysis further supported the results observed in the differential gene expression (DGE) study. Thus, first, the enrichment analysis produced a total of 482 protein sets and pathways with $p < 0.001$, four of them up-modulated—although none of them showed an FDR value below 0.25—and the remaining 478 down-modulated (**Supplementary Table 4**). We further filtered the results to analyze only those GO terms that presented a much more significant enrichment ($p < 10^{-9}$). This process left us with a total of 66 down-modulated GO terms, but none up-modulated. These 66 elements, ordered by decreasing ES, are shown in **Table 3**. Interestingly, among the most significantly enriched down-modulated pathways, we found several GO annotations referring to immune-related functionality (for instance *Interferon-Gamma-Mediated Signaling Pathway*, ES: 10.65; *Cytokine-Mediated Signaling Pathway*, ES: 5.09; or *Immune Response*, ES: 3.03), class II-related antigen presentation (like *MHC Class II Protein Complex*, ES: 30.23; *Antigen Processing And Presentation Of Exogenous Peptide Antigen Via MHC Class II*, ES: 8.08; or *Antigen Processing And Presentation Of Exogenous Peptide Antigen*, ES: 6.74), cell response to different stimuli (*Cell Surface Receptor Signaling Pathway*, ES: 2.21; *Response To Stress*, ES: 1.93; or *Cellular Response To Stimulus*, ES: 1.80) and, specially, to cell cycle and mitotic division (for instance *Condensed Chromosome Outer Kinetochore*, ES: 21.98; *Mitotic Spindle Organization*, ES: 8.79; *Microtubule Cytoskeleton Organization Involved In Mitosis*, ES: 7.48; or *Cell Cycle Checkpoint*, ES: 6.05). Our results, in line with the DGE analysis, would suggest that vitD3-Tcell are undergoing a process of transcriptomic down-modulation leading to reduced immune-related, metabolic and proliferative functionalities.

TABLE 2 | Top 50 down-modulated genes in vitD3-Tcell compared to mDC-Tcell.

GeneSymbol	GO annotation	FC vs mDC-Tcell	padj
IL13	Cytokine	−2.59	0.00082
C1orf106	NA	−2.43	0.00486
RYR2	Ligand-Gated Ion Channel	−2.35	0.00802
HLA-DQA2	Major Histocompatibility Complex Antigen	−2.25	0.03271
UBE2C	NA	−2.23	0.00000
DEPDC1	Nucleic Acid Binding	−2.23	0.00367
NEK2	Protein Kinase	−2.20	0.00206
EBI3	Cytokine; Defense/Immunity Protein	−2.18	0.00009
CCL17	Chemokine	−2.18	0.01852
AURKB	Non-Receptor Serine/Threonine Protein Kinase	−2.14	0.00006
CYP1B1	Oxygenase	−2.12	0.02334
SPC25	Enzyme Modulator	−2.11	0.01556
KIF4A	Microtubule Binding Motor Protein	−2.09	0.00001
KIF18B	Microtubule Binding Motor Protein	−2.07	0.00001
CCNB2	Kinase Activator	−2.06	0.00000
HLA-DRB5	Major Histocompatibility Complex Antigen	−2.02	0.00970
MCM10	NA	−2.01	0.00003
HIST1H3C	Histone	−2.01	0.00000
KIF15	Microtubule Binding Motor Protein	−1.97	0.00000
BIRC5	Protease Inhibitor	−1.96	0.00002
CHST3	NA	−1.95	0.01782
FAM111B	NA	−1.95	0.00000
MYBL2	NA	−1.92	0.00000
ATP8B4	Cation Transporter. Hydrolase	−1.92	0.00000
SKA1	NA	−1.91	0.00898
KIAA0101	NA	−1.91	0.00292
TK1	Nucleotide Kinase	−1.90	0.00001
HIST1H3J	Nucleic Acid Binding; Transcription Factor	−1.89	0.03504
E2F8	Nucleic Acid Binding; Transcription Factor	−1.89	0.00209
HLA-DQA1	Major Histocompatibility Complex Antigen	−1.88	0.00011
HIST1H3F	Reductase	−1.88	0.00005
RRM2	Reductase	−1.88	0.00337
GNG4	Heterotrimeric G-Protein	−1.87	0.01479
PRR11	NA	−1.86	0.00041
CEP55	NA	−1.85	0.00008
CKAP2L	NA	−1.84	0.00151
CDCA8	NA	−1.84	0.00015
HIST1H3G	Histone	−1.83	0.00000
CDK1	Non-Receptor Serine/Threonine Protein Kinase; Non-Receptor Tyrosine Protein Kinase	−1.83	0.00283
HMMR	NA	−1.83	0.00012
PKMYT1	Non-Receptor Serine/Threonine Protein Kinase	−1.83	0.00825
CCL22	Chemokine	−1.82	0.02508
CREB3L3	NA	−1.81	0.02749
CDC25A	Protein Phosphatase	−1.81	0.02454
DTL	NA	−1.81	0.00008
RAD51AP1	NA	−1.80	0.00912
ESCO2	NA	−1.79	0.01020
LIF	Cytokine	−1.78	0.00000
KIFC1	Microtubule Binding Motor Protein	−1.78	0.00000
ASB2	NA	−1.78	0.00000

Gene expression values from an RNA-seq analysis with healthy donors (n = 10). Data presented as the mean fold change (FC) of expression in vitD3-Tcell compared to mDC-Tcell. GO, Gene Ontology; mDC-Tcell, mature dendritic cell-conditioned $CD3^+CD4^+$ cells; NA, not available; padj, adjusted p-value; vitD3-Tcell, vitamin D3-induced tolerogenic dendritic cell-conditioned $CD3^+CD4^+$ cells.

DISCUSSION

In this study we analyzed the specific effect of vitD3-tolDC over autologous $CD4^+$ T cells. Thus, we switched the attention from the study of tolDC themselves—widely studied so far—to focus on the study of the functional effect that these cells develop over T cells upon their interaction. In homeostatic conditions, either depletion, inactivation and/or induction of anergy is often induced on T cells due to a lack of one or more of the three immunogenic activation signals. This causes T cells to become hyporesponsive or to die (20, 30). However, in the case of autoimmunity, where T cells are already activated and developing an immunogenic response, an antigen-specific process of tolerance induction is required. In this regard, previous *in vivo* studies with vitD3-tolDC in the EAE model showed that an antigen-specific setup—and therefore an active

TABLE 3 | Most significantly down-regulated Gene Ontology terms in vitD3-Tcell compared to mDC-Tcell.

GO category	GO term	ES	p-value	FDR
GOCOMPONENT	MHC Class II Protein Complex	30.23	5.24E-16	2.39E-13
GOCOMPONENT	Condensed Chromosome Outer Kinetochore	21.98	3.96E-10	4.81E-08
GOCOMPONENT	MHC Protein Complex	18.71	1.94E-12	5.88E-10
GOCOMPONENT	Clathrin-Coated Endocytic Vesicle Membrane	16.92	6.86E-10	7.81E-08
GOFUNCTION	Peptide Antigen Binding	16.92	6.86E-10	7.06E-07
GOPROCESS	Interferon-Gamma-Mediated Signaling Pathway	10.65	1.35E-13	1.44E-10
GOCOMPONENT	DNA Packaging Complex	10.26	5.61E-19	5.11E-16
GOPROCESS	Nuclear Chromosome Segregation	10.21	9.93E-10	2.98E-07
GOCOMPONENT	Nucleosome	9.82	2.16E-16	1.31E-13
GOPROCESS	Mitotic Spindle Organization	8.79	1.87E-11	8.07E-09
GOPROCESS	Antigen Processing And Presentation Of Exogenous Peptide Antigen Via MHC Class II	8.08	1.20E-12	8.70E-10
GOPROCESS	Regulation Of Chromosome Segregation	7.98	9.85E-14	1.24E-10
GOPROCESS	Antigen Processing And Presentation Of Peptide Or Polysaccharide Antigen Via MHC Class II	7.98	1.50E-12	9.88E-10
GOPROCESS	Antigen Processing And Presentation Of Peptide Antigen Via MHC Class II	7.98	1.50E-12	9.43E-10
GOPROCESS	Microtubule Cytoskeleton Organization Involved In Mitosis	7.48	1.93E-11	8.07E-09
GOPROCESS	Chromosome Segregation	7.21	8.19E-13	7.07E-10
GOPROCESS	Mitotic Cell Cycle	7.20	1.49E-15	2.29E-12
GOPROCESS	Nucleosome Assembly	7.08	9.78E-14	1.35E-10
GOPROCESS	Antigen Processing And Presentation Of Exogenous Peptide Antigen	6.74	1.09E-11	5.59E-09
GOPROCESS	Antigen Processing And Presentation Of Exogenous Antigen	6.62	1.57E-11	6.98E-09
GOCOMPONENT	Protein-DNA Complex	6.57	5.01E-14	1.82E-11
GOPROCESS	Antigen Processing And Presentation Of Peptide Antigen	6.32	3.73E-11	1.47E-08
GOPROCESS	Spindle Organization	6.11	2.12E-10	7.32E-08
GOPROCESS	Cell Cycle Checkpoint	6.05	2.87E-11	1.16E-08
GOPROCESS	Antigen Processing And Presentation	5.91	1.55E-11	7.15E-09
GOCOMPONENT	Midbody	5.75	9.66E-12	1.95E-09
GOPROCESS	Nucleosome Organization	5.71	1.12E-11	5.52E-09
GOPROCESS	Regulation Of Mitotic Nuclear Division	5.46	7.83E-11	2.92E-08
GOPROCESS	Regulation Of Nuclear Division	5.39	1.37E-11	6.52E-09
GOPROCESS	Cytokine-Mediated Signaling Pathway	5.09	3.61E-27	2.49E-23
GOCOMPONENT	Spindle	5.07	1.32E-10	1.85E-08
GOCOMPONENT	Chromosome	4.67	9.10E-12	2.07E-09
GOPROCESS	Mitotic Cell Cycle Process	4.64	3.48E-28	4.81E-24
GOPROCESS	Positive Regulation Of Cell Cycle Process	4.55	4.10E-11	1.57E-08
GOPROCESS	Protein-DNA Complex Assembly	4.47	7.59E-10	2.33E-07
GOPROCESS	Cell Division	4.25	5.31E-16	9.17E-13
GOPROCESS	Negative Regulation Of Cell Cycle Process	4.04	6.43E-10	2.02E-07
GOPROCESS	Regulation Of Mitotic Cell Cycle Phase Transition	3.99	1.04E-12	8.42E-10
GOPROCESS	Chromosome Organization	3.94	1.50E-12	1.04E-09
GOPROCESS	Regulation Of Cell Cycle Phase Transition	3.82	1.92E-12	1.15E-09
GOPROCESS	Cell Cycle Process	3.63	2.27E-25	1.05E-21
GOPROCESS	Cell Cycle	3.56	1.11E-12	8.53E-10
GOPROCESS	Regulation Of Cell Cycle Process	3.47	7.37E-17	1.69E-13
GOFUNCTION	Protein Heterodimerization Activity	3.44	5.36E-10	7.36E-07
GOCOMPONENT	Chromosomal Part	3.27	8.53E-20	1.55E-16
GOCOMPONENT	Nuclear Chromosome Part	3.14	3.70E-11	6.75E-09
GOPROCESS	Regulation Of Mitotic Cell Cycle	3.12	2.72E-12	1.44E-09
GOPROCESS	Negative Regulation Of Cell Cycle	3.12	2.28E-10	7.66E-08
GOPROCESS	Immune Response	3.03	2.51E-12	1.45E-09
GOPROCESS	Regulation Of Cell Cycle	2.82	6.87E-17	1.90E-13
GOPROCESS	Positive Regulation Of Immune System Process	2.68	5.07E-10	1.67E-07
GOFUNCTION	Protein Dimerization Activity	2.58	1.83E-12	3.76E-09
GOPROCESS	Immune System Process	2.36	3.18E-16	6.27E-13
GOPROCESS	Regulation Of Immune System Process	2.31	1.35E-10	4.91E-08
GOCOMPONENT	Cytoskeletal Part	2.26	7.15E-12	1.86E-09
GOPROCESS	Cell Surface Receptor Signaling Pathway	2.21	2.80E-13	2.76E-10
GOPROCESS	Response To Stress	1.93	6.04E-13	5.56E-10
GOCOMPONENT	Extracellular Region Part	1.83	1.79E-10	2.33E-08
GOCOMPONENT	Non-Membrane-Bounded Organelle	1.82	1.01E-10	1.67E-08
GOCOMPONENT	Intracellular Non-Membrane-Bounded Organelle	1.82	1.01E-10	1.53E-08
GOPROCESS	Cellular Response To Stimulus	1.80	6.19E-10	1.99E-07
GOPROCESS	Response To Stimulus	1.78	6.82E-17	2.35E-13
GOPROCESS	Signal Transduction	1.76	9.95E-14	1.14E-10

(Continued)

TABLE 3 | Continued

GO category	GO term	ES	p-value	FDR
GOPROCESS	Regulation Of Cellular Process	1.28	1.91E-10	6.76E-08
GOFUNCTION	Protein Binding	1.26	4.87E-15	2.01E-11
GOPROCESS	Cellular Process	1.15	2.64E-12	1.46E-09

Results presented as Enrichment Score (ES) values of different Gene Ontology (GO) terms in vitD3-Tcell compared to mDC-Tcell using data from an RNA-seq analysis with healthy donors (n = 10). FDR, False Discovery Rate; GOCOMPONENT, GO cellular component; GOFUNCTION, GO molecular function; GOPROCESS, GO biological process; mDC-Tcell, mature dendritic cell-conditioned $CD3^+CD4^+$ cells; NA, not available; padj, adjusted p-value; vitD3-Tcell, vitamin D3-induced tolerogenic dendritic cell-conditioned $CD3^+CD4^+$ cells.

process—is required, provided that a beneficial effect of this therapy was only observed when vitD3-tolDC were pulsed with the adequate immunogenic peptide (23, 24). Consequently, we developed an experimental model for the generation of autologous antigen-specific vitD3-tolDC and T cells from healthy donors, using an immunogenic peptide presented *via* class II MHC with the aim to reproduce antigen presentation in the context of $CD4^+$-mediated autoimmune diseases. In this regard, we selected TT for its compliance with this feature—since the vaccination against TT is included in European health systems—which also allowed us to use healthy donors instead of patients of a determined autoimmune disease, thus eliminating disease-conditioned immune variations.

All in all, our approach aimed to be as versatile as possible, and to serve as a preliminary study for future research, with the idea that the immunogenic peptide/s might be replaced depending on the disease of interest, as well as, of course, using patient cells instead of healthy donors. On the one hand, for autoimmune diseases with identified autoantigens, this decision would be trivial. On the other hand, for those conditions in which autoimmune antigens are yet to be identified, the experimental design should be considered case by case. For instance, using autologous synovial fluid as a source for autoantigens to load tolDC has provided promising results in rheumatoid arthritis (31), so analog workaround solutions could be taken into account.

After validating our experimental setup—meaning that vitD3-tolDC-TT were able to induce an antigen-specific response—we focused on the study of the actual phenotypic, functional and transcriptomic modulations induced by vitD3-tolDC. First, the analysis of the phenotype of T $CD4^+$ cells evidenced that their interaction with vitD3-tolDC-TT caused a relative reduction in the activation of these cells. More importantly, a switch in the immune response of these cells towards a more immunoregulatory profile was induced, with a reduction in the prevalence of T_H1 memory subpopulations. These results were further supported by the decrease of IFN-γ production in the autologous co-culture supernatants, consequently supporting that vitD3-tolDC were inducing a switch towards a more anti-inflammatory immune profile, in line with previous findings in the literature (16–18).

When we deepened into the analysis of the vitD3-tolDC-mediated transcriptomic profile of T cells, we observed several genes and GO terms regulated in line with the abovementioned phenotypical and functional switch towards a less activated an more immunoregulatory profile; for instance, a down-modulation of *STAT1* gene and the interferon-gamma-mediated signaling pathway was observed, which on the other hand supported the robustness of our RNA-seq study. Beyond this, the results pointed towards a generalized down-modulation of the transcriptomic profile of vitD3-Tcell that could either respond to an induction of T $CD4^+$ cell hyporesponsiveness or even to a process of clonal deletion. On the one hand, the down-modulation of genes and pathways involved in crucial cellular mechanisms—in particular those related to cell proliferation, mitosis, cell cycle and response to immune stimuli, some of them never reported before—could be explained by both of these processes. However, on the other hand, the lack of induction of cell death and apoptotic-related pathways makes clonal deletion very unlikely to be happening. Therefore, our results suggest that the antigen-specific interaction of vitD3-tolDC with autologous T $CD4^+$ cells is mediating, in fact, an induction of hyporesponsiveness over these cells. Furthermore, previous studies from our group already pointed in this direction (16), a biological situation that, potentially, might lead to the abrogation of an autoimmune immunogenic response in patients. Moreover, our current results provide evidence that these modulations are taking place at the transcriptomic level in T $CD4^+$ cells, indicating that the antigen-specific modulation induced by vitD3-tolDC is deeper than expected and, in consequence, probably also long-lasting.

Unfortunately, the lack of strongly up-regulated genes among the protein-coding RNA transcripts did not allow us to point towards many clear candidate biomarkers that might become indicators of the response of T cells upon their interaction with vitD3-tolDC to monitor patients in clinical trials. One of the most relevant exceptions came given by *JUNB* gene, encoding a member of the AP-1 family of transcription factors. In experimental models, this gene has been reported to be crucial in maintaining Treg suppressive function (32), although it is also apparently involved in the induction and maintenance of IL-23-related pathogenicity of T_H17 cells (33, 34). However, neither of these functionalities seems to fit in our model based on our results, since neither T_H17 nor Treg induction was evidenced. Consequently, it would be interesting to elucidate the specific role of JunB in our experimental setting, and to what extent small changes in its expression can actually be sufficient or not for the induction and maintenance of immune tolerance. Furthermore, if this hypothesis proves to be valid, either *JUNB* and/or other related genes might also constitute potential biomarkers of response to vitD3-tolDC treatment in the clinic. It is also worth noting that we may have overlooked other potential biomarkers that might be found among the non-protein-coding and alternate splicing RNA transcripts. Although this

possibility, if true, would have a limited functional value in our experimental model, it could be addressed in future studies.

In addition, our results did not allow us to reach any conclusion regarding a potential induction of anergy, and, as discussed above, they also rule out any kind of Treg or Tr1 response. Even though our previous *in vivo* experiments with the murine EAE model pointed towards an induction of Treg mediated by vitD3-tolDC (23, 24), we have observed that, at least in this experimental setting, this is not the case with human cells. These results are in line with what previous studies from both our group and other authors have already reported (13, 16, 35), although there seems to be some controversy (36, 37). However, it is also worth mentioning that in these reports, Treg induction was only observed after two rounds of stimulation of T cells, which might explain why we have not detected it. This is definitely something to be taken into account, since Treg induction is, undoubtedly, one of the main mechanisms for the induction of immune tolerance of tolDC and other antigen presenting cell approaches (38, 39). Indeed, Treg themselves, when expanded *in vitro*, present a huge therapeutic potential as a cell therapy for autoimmune diseases in humans (40). Consequently, the transcriptomic study of vitD3-tolDC-induced Treg should probably be addressed separately in future studies, since two rounds of T cell stimulation might have masked some of the results that we have reported here.

Our current study presents some limitations. First, since we focused on the study of $CD4^+$ T cells alone, we were naturally omitting the potential modulation that vitD3-tolDC might be mediating through other subpopulations, such as regulatory B cells or regulatory NK cells. Furthermore, the election of the timepoint for the RNA-seq analysis intrinsically establishes another limitation, which is the status of the transcriptomic profile at different timepoints of the co-culture. However, our selection came based on the phenotypical and functional results shown in the study, which evidence that, by day 5 of the co-culture, there is a significant and differential modulation mediated by vitD3-tolDC over T $CD4^+$ cells. Consequently, even though it is true that other timepoints might provide valuable additional information, we think that our election provided the best compromise, and a full time-course characterization of the antigen-specific transcriptomic changes induced by vitD3-tolDC will be addressed in future studies. On the other hand, we cannot fully discard the presence of non-antigen-specific $CD4^+$ cells by the time the cell sorting was performed. However, even with a residual amount of non-antigen-specific T cells, the obtained results were consistent not only within the different techniques, but also with the literature, supporting our findings.

In conclusion, our results evidence that vitD3-tolDC are inducing a strong antigen-specific transcriptomic down-modulation over autologous T $CD4^+$ cells, with a reduced ability to respond to immune- and non-immune-related stimuli. Consequently, it constitutes one of the first attempts to understand the changes that T cells are undergoing at the transcriptomic level upon an antigen-specific interaction with a tolerogenic cell product, such as vitD3-tolDC. In that regard, we identified several specific genes and pathways selectively down-modulated, as well as the induction of *JUNB*, which might constitute a putative biomarker of the modulation mediated by vitD3-tolDC over $CD4^+$ T cells and, consequently, a potential biomarker to monitor the effect of vitD3-tolDC after their administration to patients. Therefore, the results presented in this article allowed us to better understand the process of T cell hyporesponsiveness at the molecular level and, more importantly, to set the path for future studies to fully elucidate the specific processes that are taking place in one of the most important mechanisms that the promising tolDC-based therapies can trigger in order to restore tolerance in autoimmune diseases.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE128816.

ETHICS STATEMENT

This study was reviewed and approved by the Germans Trias i Pujol Hospital Ethical Committee. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EM-C, JN-B and MM conceived the experiments. JN-B and MM performed the cell cultures and the cell sortings, and prepared the samples for the RNA-seq analysis. JN-B and MM analyzed the results. EM-C, JN-B and MM interpreted the results. JN-B wrote the manuscript. AT-S, BQ-S, CR-T, EM-C, JN-B, and MM reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partially supported by projects PI14/01175, PI16/01737 and PI17/01521, integrated in the *Plan Nacional de I+D+I* and co-supported by the ISCIII-*Subdirección General de Evaluación* and the *Fondo Europeo de Desarrollo Regional* (FEDER), and by grant 140191 from IWT-TBM (Belgium). JN-B was beneficiary of a FI-DGR PhD contract from AGAUR (*Agència de Gestió d'Ajuts Universitaris i de Recerca*), supported by the Government of Catalonia (02/2015-01/2018). The contracts of JN-B and MM have been partially supported by grant 140191 from IWT-TBM. The authors are members of a consolidated group recognized by AGAUR (2017 SGR 103), supported by the Government of Catalonia. This work has been supported by positive discussion through A FACTT network (COST Action BM1305: www.afactt.eu) and project

779316–ReSToRe (www.h2020restore.eu). COST and ReSToRe are supported by the EU Framework Programme Horizon 2020.

ACKNOWLEDGMENTS

We want to thank Marco A. Fernández-Sanmartín and Gerard Requena, from the IGTP Flow Cytometry Platform, Edurne Pedrosa, Verónica Guirao and Ingrid Liarte, from the IGTP Biobank, Lauro Sumoy and Raquel Pluvinet, from the IGTP High Content Genomics and Bioinformatics Unit, and, specifically, Jorge Carrascal and all the members of the IGTP Immunology Division for their support and technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.599623/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Phenotype and functionality of DC. **(A)** Gating strategy. **(B)** Surface expression of CD86 (FITC) and HLA-DR (V500) on immature DC (iDC), mature DC (mDC), tetanus toxin (TT)-loaded mDC (mDC-TT), vitamin D3-induced tolerogenic DC (vitD3-tolDC) and TT-loaded vitD3-tolDC (vitD3-tolDC-TT) (n = 8). Data presented as relative percentage of median fluorescence intensity (MFI) normalized *versus* mDC, measured by flow cytometry. In all cases, DC populations were $CD11c^+$ **(C)** Allogeneic proliferation of PBMC from healthy donors co-cultured with either iDC, mDC, mDC-TT, vitD3-tolDC or vitD3-tolDC-TT (n = 14) in a DC : PBMC 1:20 ratio. Data presented as relative percentage of induced proliferation compared to mDC, measured as tritiated thymidine incorporation after 18 h. Six replicated measurements of each condition were performed. Error bars corresponding to SEM. ns = not significant; * $p < 0.05$. Friedman test with Dunn's correction. **(D)** Gating strategy for the sorting of autologous T $CD4^+$ cells.

SUPPLEMENTARY FIGURE 2 | Assignment and functional classification of the total reads from the RNA-seq study of T $CD4^+$ cells co-cultured with autologous antigen-specific DC. **(A)** Assignment of the total reads from the RNA-seq analysis to known RNA classes. **(B)** Classification of the assigned reads into known RNA functionalities.

SUPPLEMENTARY TABLE 3 | Up and down-modulated genes in vitD3-Tcell compared to mDC-Tcell.

SUPPLEMENTARY TABLE 4 | Differentially enriched Gene Ontology terms in vitD3-Tcell compared to mDC-Tcell.

REFERENCES

1. Ten Brinke A, Hilkens CMU, Cools N, Geissler EK, Hutchinson JA, Lombardi G, et al. Clinical Use of Tolerogenic Dendritic Cells-Harmonization Approach in European Collaborative Effort. *Mediators Inflammation* (2015) 2015:471719. doi: 10.1155/2015/471719
2. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu Y-J, et al. Immunobiology of Dendritic Cells. *Annu Rev Immunol* (2000) 18:767–811. doi: 10.1146/annurev.immunol.18.1.767
3. Mueller DL. Mechanisms maintaining peripheral tolerance. *Nat Immunol* (2010) 11:21–7. doi: 10.1038/ni.1817
4. Ganguly D, Haak S, Sisirak V, Reizis B. The role of dendritic cells in autoimmunity. *Nat Rev Immunol* (2013) 13:566–77. doi: 10.1038/nri3477
5. Hopp A-K, Rupp A, Lukacs-Kornek V. Self-Antigen Presentation by Dendritic Cells in Autoimmunity. *Front Immunol* (2014) 5:55. doi: 10.3389/fimmu.2014.00055
6. Schinnerling K, García-González P, Aguillón JC. Gene expression profiling of human monocyte-derived dendritic cells – searching for molecular regulators of tolerogenicity. *Front Immunol* (2015) 6:528. doi: 10.3389/fimmu.2015.00528
7. Navarro-Barriuso J, Mansilla MJ, Martínez-Cáceres EM. Searching for the Transcriptomic Signature of Immune Tolerance Induction—Biomarkers of Safety and Functionality for Tolerogenic Dendritic Cells and Regulatory Macrophages. *Front Immunol* (2018) 9:2062. doi: 10.3389/fimmu.2018.02062
8. Adorini L, Penna G, Giarratana N, Roncari A, Amuchastegui S, Daniel KC, et al. Dendritic cells as key targets for immunomodulation by Vitamin D receptor ligands. *J Steroid Biochem Mol Biol* (2004) 89–90:437–41. doi: 10.1016/j.jsbmb.2004.03.013
9. Penna G, Roncari A, Amuchastegui S, Daniel KC, Berti E, Colonna M, et al. Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+ regulatory T cells by 1,25-dihydroxyvitamin D3. *Blood* (2005) 106:3490–7. doi: 10.1182/blood-2005-05-2044
10. Adorini L, Penna G. Induction of tolerogenic dendritic cells by vitamin D receptor agonists. *Handb Exp Pharmacol* (2009) 188:251–73. doi: 10.1007/978-3-540-71029-5_12
11. Pedersen AW, Holmstrøm K, Jensen SS, Fuchs D, Rasmussen S, Kvistborg P, et al. Phenotypic and functional markers for 1α,25-dihydroxyvitamin D3-modified regulatory dendritic cells. *Clin Exp Immunol* (2009) 157:48–59. doi: 10.1111/j.1365-2249.2009.03961.x
12. Széles L, Keresztes G, Töröcsik D, Balajthy Z, Krenács L, Póliska S, et al. 1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype. *J Immunol Baltim Md 1950* (2009) 182:2074–83. doi: 10.4049/jimmunol.0803345
13. Naranjo-Gómez M, Raïch-Regué D, Oñate C, Grau-López L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. *J Transl Med* (2011) 9:89. doi: 10.1186/1479-5876-9-89
14. Ferreira GB, Kleijwegt FS, Waelkens E, Lage K, Nikolic T, Hansen DA, et al. Differential protein pathways in 1,25-dihydroxyvitamin d(3) and dexamethasone modulated tolerogenic human dendritic cells. *J Proteome Res* (2012) 11:941–71. doi: 10.1021/pr200724e
15. Raïch-Regué D, Naranjo-Gómez M, Grau-López L, Ramo C, Pujol-Borrell R, Martínez-Cáceres E, et al. Differential effects of monophosphoryl lipid A and cytokine cocktail as maturation stimuli of immunogenic and tolerogenic dendritic cells for immunotherapy. *Vaccine* (2012) 30:378–87. doi: 10.1016/j.vaccine.2011.10.081
16. Raïch-Regué D, Grau-López L, Naranjo-Gómez M, Ramo-Tello C, Pujol-Borrell R, Martínez-Cáceres E, et al. Stable antigen-specific T-cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients. *Eur J Immunol* (2012) 42:771–82. doi: 10.1002/eji.201141835
17. Ferreira GB, Vanherwegen A-S, Eelen G, Gutiérrez ACF, Van Lommel L, Marchal K, et al. Vitamin D3 Induces Tolerance in Human Dendritic Cells by Activation of Intracellular Metabolic Pathways. *Cell Rep* (2015) 10:711–25. doi: 10.1016/j.celrep.2015.01.013
18. Malaguarnera L, Marsullo A, Zorena K, Musumeci G, Di Rosa M. Vitamin D3 regulates LAMP3 expression in monocyte derived dendritic cells. *Cell Immunol* (2017) 311:13–21. doi: 10.1016/j.cellimm.2016.09.013
19. Xing N, Maldonado ML L, Bachman LA, McKean DJ, Kumar R, Griffin MD. Distinctive dendritic cell modulation by vitamin D(3) and glucocorticoid pathways. *Biochem Biophys Res Commun* (2002) 297:645–52. doi: 10.1016/S0006-291X(02)02262-3
20. Maldonado RA, von Andrian UH. Chapter 4 - How Tolerogenic Dendritic Cells Induce Regulatory T Cells. In: FW Alt, KF Austen, T Honjo, F Melchers, JW Uhr, ER Unanue, editors. *Advances in Immunology*. USA: Academic Press (2010). p. 111–65. doi: 10.1016/B978-0-12-380995-7.00004-5
21. Maggi J, Schinnerling K, Pesce B, Hilkens CM, Catalán D, Aguillón JC. Dexamethasone and Monophosphoryl Lipid A-Modulated Dendritic Cells Promote Antigen-Specific Tolerogenic Properties on Naive and Memory CD4+ T Cells. *Front Immunol* (2016) 7:359. doi: 10.3389/fimmu.2016.00359
22. Navarro-Barriuso J, Mansilla MJ, Naranjo-Gómez M, Sánchez-Pla A, Quirant-Sánchez B, Teniente-Serra A, et al. Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3,

dexamethasone and rapamycin. *Sci Rep* (2018) 8:14985. doi: 10.1038/s41598-018-33248-7
23. Mansilla MJ, Sellès-Moreno C, Fàbregas-Puig S, Amoedo J, Navarro-Barriuso J, Teniente-Serra A, et al. Beneficial effect of tolerogenic dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. *CNS Neurosci Ther* (2015) 21:222–30. doi: 10.1111/cns.12342
24. Mansilla MJ, Contreras-Cardone R, Navarro-Barriuso J, Cools N, Berneman Z, Ramo-Tello C, et al. Cryopreserved vitamin D3-tolerogenic dendritic cells pulsed with autoantigens as a potential therapy for multiple sclerosis patients. *J Neuroinflamm* (2016) 13:113. doi: 10.1186/s12974-016-0584-9
25. Navarro-Barriuso J, Mansilla MJ, Quirant-Sánchez B, Ardiaca-Martínez A, Teniente-Serra A, Presas-Rodríguez S, et al. MAP7 and MUCL1 are biomarkers of vitamin D3-induced tolerogenic dendritic cells in multiple sclerosis patients. *Front Immunol* (2019) 10:1251. doi: 10.3389/fimmu.2019.01251
26. Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, et al. Ensembl 2018. *Nucleic Acids Res* (2018) 46:D754–61. doi: 10.1093/nar/gkx1098
27. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* (2014) 15:550. doi: 10.1186/s13059-014-0550-8
28. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinf* (2009) 10:48. doi: 10.1186/1471-2105-10-48
29. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the Human Immunology Project. *Nat Rev Immunol* (2012) 12:191–200. doi: 10.1038/nri3158
30. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. *Lymphocytes and the Cellular Basis of Adaptive Immunity. Mol Biol Cell* (2002). Available at: https://www.ncbi.nlm.nih.gov/books/NBK26921/ (Accessed February 21, 2019).
31. Bell GM, Anderson AE, Diboll J, Reece R, Eltherington O, Harry RA, et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann Rheum Dis* (2016) 76:227–34. doi: 10.1136/annrheumdis-2015-208456
32. Koizumi S, Sasaki D, Hsieh T-H, Taira N, Arakaki N, Yamasaki S, et al. JunB regulates homeostasis and suppressive functions of effector regulatory T cells. *Nat Commun* (2018) 9:5344. doi: 10.1038/s41467-018-07735-4
33. Hasan Z, Koizumi S, Sasaki D, Yamada H, Arakaki N, Fujihara Y, et al. JunB is essential for IL-23-dependent pathogenicity of Th17 cells. *Nat Commun* (2017) 8:15628. doi: 10.1038/ncomms15628
34. Carr TM, Wheaton JD, Houtz GM, Ciofani M. JunB promotes Th17 cell identity and restrains alternative CD4+ T-cell programs during inflammation. *Nat Commun* (2017) 8:301. doi: 10.1038/s41467-017-00380-3
35. Sochorová K, Budinský V, Rožková D, Tobiasová Z, Dusilová-Sulková S, Špíšek R, et al. Paricalcitol (19-nor-1,25-dihydroxyvitamin D2) and calcitriol (1,25-dihydroxyvitamin D3) exert potent immunomodulatory effects on dendritic cells and inhibit induction of antigen-specific T cells. *Clin Immunol* (2009) 133:69–77. doi: 10.1016/j.clim.2009.06.011
36. Unger WWJ, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol* (2009) 39:3147–59. doi: 10.1002/eji.200839103
37. Vanherwegen A-S, Eelen G, Ferreira GB, Ghesquière B, Cook DP, Nikolic T, et al. Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. *J Steroid Biochem Mol Biol* (2019) 187:134–45. doi: 10.1016/j.jsbmb.2018.11.011
38. Boks MA, Kager-Groenland JR, Haasjes MSP, Zwaginga JJ, van Ham SM, ten Brinke A. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction–a comparative study of human clinical-applicable DC. *Clin Immunol Orlando Fla* (2012) 142:332–42. doi: 10.1016/j.clim.2011.11.011
39. Riquelme P, Haarer J, Kammler A, Walter L, Tomiuk S, Ahrens N, et al. TIGIT + iTregs elicited by human regulatory macrophages control T cell immunity. *Nat Commun* (2018) 9:2858. doi: 10.1038/s41467-018-05167-8
40. Marek-Trzonkowska N, Myśliwiec M, Dobyszuk A, Grabowska M, Derkowska I, Juścińska J, et al. Therapy of type 1 diabetes with CD4(+) CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. *Clin Immunol Orlando Fla* (2014) 153:23–30. doi: 10.1016/j.clim.2014.03.016

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 17 February 2021
doi: 10.3389/fimmu.2021.607315

IL10- and IL35-Secreting MutuDC Lines Act in Cooperation to Inhibit Memory T Cell Activation Through LAG-3 Expression

Marianna M. Koga[1†], Adrien Engel[1], Matteo Pigni[1], Christine Lavanchy[1], Mathias Stevanin[1], Vanessa Laversenne[2], Bernard L. Schneider[2,3] and Hans Acha-Orbea[1]*

[1] Department of Biochemistry, Center of Immunity and Infection Lausanne, University of Lausanne, Lausanne, Switzerland, [2] Brain Mind Institute, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, [3] Bertarelli Platform for Gene Therapy, École Polytechnique Fédérale de Lausanne, Geneva, Switzerland

OPEN ACCESS

Edited by:
Femke Broere,
Utrecht University, Netherlands

Reviewed by:
Huanfa Yi,
Jilin University, China
Muriel Moser,
Université Libre de Bruxelles, Belgium

***Correspondence:**
Hans Acha-Orbea
hans.acha-orbea@unil.ch

†Present address:
Marianna M. Koga,
Institute of Materials, School of Engineering, École Polythechnique Fédérale de Lausanne, Lausanne, Switzerland

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: 16 September 2020
Accepted: 26 January 2021
Published: 17 February 2021

Citation:
Koga MM, Engel A, Pigni M, Lavanchy C, Stevanin M, Laversenne V, Schneider BL and Acha-Orbea H (2021) IL10- and IL35-Secreting MutuDC Lines Act in Cooperation to Inhibit Memory T Cell Activation Through LAG-3 Expression. Front. Immunol. 12:607315. doi: 10.3389/fimmu.2021.607315

Dendritic cells (DCs) are professional antigen-presenting cells involved in the initiation of immune responses. We generated a tolerogenic DC (tolDC) line that constitutively secretes interleukin-10 (IL10-DCs), expressed lower levels of co-stimulatory and MHCII molecules upon stimulation, and induced antigen-specific proliferation of T cells. Vaccination with IL10-DCs combined with another tolDC line that secretes IL-35, reduced antigen-specific local inflammation in a delayed-type hypersensitivity assay independently on regulatory T cell differentiation. In an autoimmune model of rheumatoid arthritis, vaccination with the combined tolDCs after the onset of the disease impaired disease development and promoted recovery of mice. After stable memory was established, the tolDCs promoted CD4 downregulation and induced lymphocyte activation gene 3 (LAG-3) expression in reactivated memory T cells, reducing T cell activation. Taken together, our findings indicate the benefits of combining anti-inflammatory cytokines in an antigen-specific context to treat excessive inflammation when memory is already established.

Keywords: dendritic cells, IL-10, IL-35, LAG-3, tolerogenic DCs

INTRODUCTION

Dendritic cells (DC) are widely recognized as inducers of adaptive immune responses, modulating the balance between tolerance and immunity. To do so, they rely on the ability to sense the environment upon antigen uptake, migrate, and translate the signals met, not only through upregulation of MHC and co-stimulatory molecules, but also secreting cytokines and inflammatory mediators. Thus, during antigen presentation, DCs are able to activate T cells and direct their fate based on the quality of the signals they expose (1). However, in the context of autoimmune diseases, where exceeding immune activation against self-antigens takes place, it is preferable that the immune response is shifted toward tolerance. Thus, the manipulation of DCs can be advantageous as they represent key players in the development of regulatory responses.

Tolerogenic DCs (tolDCs) are essential for the maintenance of central and peripheral tolerance. They are able to induce clonal T cell deletion, T cell anergy, and regulatory T cell differentiation. DCs can further restrain memory and effector T cell responses due to impaired or sustained antigen presentation, insufficient co-stimulation, and

secretion of large amounts of anti-inflammatory mediators (2–5). TolDC differentiation can be favored by many different suppressive factors, like anti-inflammatory cytokines (e.g., TGF-β, IL-10), immunomodulatory drugs (e.g., corticosteroids), vitamin D, and other substances (6, 7). However, the stability of tolDCs and the difficulty in achieving a definitive and efficient induction protocol are issues that still need to be addressed.

The MutuDC1 cell line ($CD8\alpha^+$ murine tumor DC line) consists of an immortalized cell line generated through culture of splenic DC tumors from transgenic mice. They were developed and described by our group a few years ago and their functional and phenotypical features resemble the splenic conventional DC1s (cDC1) (8). MutuDC1s are easy to culture *in vitro* and their stability allows further transformation through lentiviral transduction system (9). Therefore, the MutuDC1s represent a great tool to explore the effects caused by the overexpression of immunosuppressive molecules.

We have previously described the generation of a genetically modified MutuDC1 line that constitutively secretes the anti-inflammatory cytokine IL-35 (IL35-DCs). The overexpression of IL-35 in the IL35-DCs was shown to strongly regulate antigen-specific $CD4^+$ and $CD8^+$ T cell responses *in vitro* and *in vivo*, conferring a tolDC phenotype to the MutuDC1s. In addition, vaccination with IL35-DCs both prevented and ameliorated disease severity on experimental autoimmune encephalitis (EAE), indicating an encouraging approach for treating autoimmune diseases (10). Employing the same approach used to generate the IL35-DC, we have developed a new MutuDC1 cell line that constitutively expresses high amounts of IL-10 (IL10-DC). IL-10 is a potent anti-inflammatory cytokine naturally produced by antigen presenting cells (APCs), B cells, eosinophils, mast cells, and many subsets of $CD4^+$ and $CD8^+$ T cells upon activation. It has a broad and strong effect on DC function, inhibiting their capacity to produce pro-inflammatory cytokines, upregulating MHC II and co-stimulatory molecules, and impairing their antigen-presenting function (11). The association of IL-10 with other cytokines like the pleiotropic TGF-β was shown to potentiate their individual anti-inflammatory features, leading to the induction of robust regulatory cells in an antigen-specific context (12). This fact indicates that anti-inflammatory cytokines could act in synergy to mediate a tolerogenic response in excessively inflammatory pathologies.

The combination of IL-10 and IL-35 secreted by tumor-infiltrating regulatory T cells was shown to induce the expression of the inhibitory receptors TIM-3, LAG-3, TIGIT, and 2B4, driving intratumoral $CD8^+$ T cells to exhaustion (13). LAG-3 is an inhibitory co-receptor involved in controlling excessive activation after persistent antigen exposure. Its expression was observed to play a suppressive role in murine autoimmune disease models of myocarditis, type-1 diabetes, and EAE (14–17), indicating that LAG-3 expression might be modulated by both IL-10 and IL-35 also during $CD4^+$ T cell activation.

In this work, we describe our new tolerogenic murine DC line that secretes high amounts of IL-10. We show that when applied in combination with the IL35-DC line, they cooperate to induce antigen-specific tolerance in overly inflamed conditions. Moreover, we show that this cooperation induced the upregulation of LAG-3 expression in memory T cells, dampening the immune response.

MATERIALS AND METHODS

Mice

OT-I/ $Rag^{-/-}$, OT-II, and $CD11b^{-/-}$ mice were bred and kept in our specific pathogen free animal facility. C57BL/6 mice were purchased from Harlan laboratories and kept under the same conditions as mentioned. For all experiments, 8–12-week-old female mice were used, except for the CIA protocol where $CD11b^{-/-}$ sex-matched groups were formed. All experimental procedures were performed in accordance with the Swiss Federal Legislation and approved by the Cantonal Veterinary Office (license number VD.3324).

Generation of the IL10-Secreting Mutu DC Line (IL10-DC)

The murine tumor DC1 (MutuDC1) line was derived from splenic tumors of transgenic CD11c:SV40LgT C57BL/6 female mouse (8, 18) and the generation and characterization of the IL35-DC line was previously described by Haller et al. (10). For the generation of the IL10-DC line, the Il10 gene was obtained from the cDNA of MutuDC1s stimulated with CpG (1 μM) and amplified by PCR using the following primers 5′-GCC ACCAT-GCCTGGCTCAGCACTG-3′ (forward) and 5′-GAT CGTCGACTTAGCTTTTCATTTTGAT-CATCAT-3′ (reverse) (synthesized by Invitrogen). The amplified DNA fragments were loaded in 1% Sea Kem GTG agarose gel (Lonza) and purified with the Wizard SV Gel and PCR Clean-up system (Promega) according to the supplier's instructions. The Il10 gene was inserted into the lentiviral vector (pWP-SIN-cPPT-WPRE)-CMV-IRES-GFP and lentiviral particles either empty or containing the IL-10 expression vector (with the GFP reporter) were produced by 293T HEK cells through a second generation transduction system using pMD2G and psPAX2 as packaging vectors. MutuDC1s were stably transduced with the IL-10 lentiviral particles generating the IL10-DC line, or with the empty vector, generating the Mock-DC line. To confirm the transgene expression, protein production was confirmed by FACS (GFP expression and IL-10 expression in GFP^+ cells) and ELISA. The MutuDC1 lines were cultured in IMDM-Glutamax (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS Good, PAN-Biotech), 10 mM Hepes (GIBCO), 50 μM β-Mercaptoethanol (GIBCO), 50 U/mL of penicillin, and 50 μg /mL streptomycin (BioConcept) at 37°C in a humidified 5% CO_2 atmosphere.

Cell Engineering and Encapsulation for Device Implantation

The murine myoblast C2C12 cell line was obtained from the American Type Culture Collection (ATCC) and cultured with DMEM (GIBCO) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) (BioConcept). To generate the C2C12 cell lines expressing IL-10 or IL-35, the Il10 gene was inserted into the lentiviral vector pRDI277 (kindly

provided by Prof. Richard Iggo, Bergonié Cancer Institute, University of Bordeaux), under the control of the CMV promoter. The Il35 construct (Ebi3 and p35 linked by Gly4Ser) was cloned into the lentiviral expression vector pCDH-CMV-MCS-EF1α-RFP+Puro (System Biosciences). Lentiviral particles were generated by transfection of 293T HEK cells using pVSV-G and psPAX as packaging vectors in serum-free DMEM, in the presence of 1 μg of polyethylenimine/μg DNA. The viral-containing supernatants were collected and filtered (0.22 μM) after 24 h. C2C12 cells were stably transduced with the lentiviral particles and selected for puromycin resistance. Transgene expression was confirmed by FACS and qPCR.

Cell preparation and encapsulation of bioactive cellular implants were previously described (19). Briefly, cells were harvested with Trypsine-EDTA solution and cell suspensions were mixed with PEG gel premix and coagulation factor XIIIa immediately before the loading of 3×10^6 cells (250 μL) into the cell encapsulation devices. The devices were placed on a rocking platform until hydrogel crosslinking was complete, and then sealed with polymerizing medical-grade glue (Loctite, Henkel). The devices were maintained in DMEM for 24 h under cell culture conditions and washed with PBS before subcutaneous (s.c.) implantation in the back of mice. Surgeries were performed under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia and mice recovered in their home cages. Analgesia was provided by a s.c. injection of Buprenorphine (0.5 mg/kg) 24 h after surgery.

Organ Collection and Processing

Blood was obtained by cardiac puncture, left at room temperature for 30 min and centrifuged for 10 min at 2,000 × g in a refrigerated centrifuge. Sera were collected and stored at −70°C. Draining lymph nodes (DLNs) and spleens were mashed through 40 μm cell strainers. For OT-I, OT-II, and memory assays, T cells were magnetically isolated using the EasySep Mouse $CD8^+$ T or $CD4^+$ T Cell Isolation Kits (STEMCELL Technologies), following manufacturer's protocols. For the other experimental protocols, cells were treated with ACK lysis buffer (NH4Cl 0.155 M, KHCO3 0.01 M, EDTA 0.1 mM) before they were seeded in culture plates and re-stimulated *ex vivo*.

In vitro OT-I and OT-II Proliferation Assays

10^4 MutuDCs were seeded in U-botton 96-well plates and pulsed with different concentrations of the ovalbumin peptides SIINFEKL (OVA257-264) (OT-I) or OVA329-337 (OT-II) for 4 h and washed. $CD8^+$ or $CD4^+$ cells isolated from OT-I/$Rag^{-/-}$ or OT-II mice, respectively, were labeled with 5 μM of the eFluor670 (ThermoFisher) or with Tag-it Violet (Biolegend) proliferation dyes. 10^5 T cells were then co-cultured with DCs for 72 h.

Delayed-Type Hypersensitivity (DTH) Assay

C57BL6 mice were immunized against OVA (50 μg – Grade IV, Sigma Aldrich) in Complete Freund's Adjuvant (CFA – InvivoGen). After 7 days, MutuDCs were pulsed with 100 μg/mL of OVA overnight, washed with PBS twice, and 3×10^6 cells were transferred to immunized mice by intraperitoneal (i.p.) injection. When IL10-DCs and IL35-DCs were transferred in combination, they were mixed only a few minutes before the injection, at 1:1 ratio. One week later, mice were challenged with 25 μL of heat-aggregated OVA (20 mg/mL – 500 μg/animal) in one footpad and the same volume of PBS was injected in the contralateral footpad as a control. Footpad thickness was measured with a dial thickness gauge (Mitutoyo) multiple times for 72 h. Blood, lymph nodes and spleen were collected and processed as mentioned above. Total cells were re-stimulated *ex vivo* with 100 μg/mL OVA for 24 h.

Collagen-Induced Arthritis (CIA)

Chicken collagen type II (CII, Sigma Aldrich) emulsified in CFA (InvivoGen) was prepared as previously described (20) and injected intradermally at the base of tail of the CIA-susceptible $CD11b^{-/-}$ mice. Mice were assessed every day for redness and swelling of limbs or ankle and scored from 1 to 4: (1) erythema and light swelling confined to 1 joint; (2) erythema and mild swelling in one joint or more; (3) erythema and moderate swelling confined to 1 joint; (4) erythema and severe swelling involving multiple joints, joint malformation or ankylosis. No boost of CII was given as mice started scoring positive for the disease as early as 2 weeks after the immunization. In one setup, tolerogenic implants were inserted 1 week after immunization; in another, when around 80% of mice scored at least 1, 5×10^6 MutuDCs pulsed with CII (100 μg/mL) overnight were transferred i.p. When IL10-DCs and IL35-DCs were transferred in combination, they were mixed only a few minutes before the injection, at 1:1 ratio. DC transfer was repeated 2 days later and mice were observed for another 7 days, then euthanized. Blood, lymph nodes and spleen were collected and processed as mentioned above. Total cells from lymph nodes and spleen were re-stimulated with 100 μg/mL of CII *ex vivo* for 24 h.

T Cell Memory Assays

C57BL6 mice were immunized against OVA (Grade IV, Sigma Aldrich) in CFA (InvivoGen) (50 μg in a total of 100 μL of emulsion per mouse), and boosted after 1 week either with OVA (same concentration) in Incomplete Freund's Adjuvant (IFA, Invitrogen), or with OVA-pulsed MutuDCs (ctrl-DCs). Fourteen days later, spleens were collected and processed as above described. Isolated T cells were labeled with 5 μM of Tag-it Violet proliferation dye (Biolegend). MutuDCs were pulsed overnight with OVA (100 μg/mL), washed twice with PBS, and seeded 96-well plates. DCs and T cells were kept in co-culture for 3 days (10^4:10^5 cells per well, respectively).

ELISA for Cytokine and Antibody Detection

For IL-10 cytokine detection, a specific ELISA kit was used according to manufacturer's instructions (BD Biosciences). To determine OVA- or CII-specific antibodies, plates were coated overnight at 4°C with 20 μg/mL of the appropriate protein in PBS. Plates were washed three times with wash buffer (0.05% Tween-20 in PBS), and blocked with assay diluent (PBS containing 10% heat-inactivated FBS) for 1 h. Mice sera samples were serial diluted in assay diluent and added to plates after three more washes. Following a 2 h incubation at room temperature (RT), plates were washed five times, and anti-IgG1 (clone 2H12B4, Chondrex) or anti-IgG2a (clone 1F10C2, Chondrex)

conjugated with peroxidase were used as secondary antibodies. Plates were incubated for 1 h RT and washed seven times. TMB Substrate (Thermo Scientific) was added and plates were left for 30 min RT in the dark. Colorimetric reaction was stopped by the addition of 2 N H_2SO_4. Absorbance was acquired at 450 nM in the microplate reader (Ledetect 96, LabExim). Absorbance sample values were considered after subtracting values of wells incubated with fresh serum from naïve mice in the same dilutions or incubated with assay diluent. Data are shown in optical density (OD) units.

Flow Cytometry Analysis

The fluorochrome-conjugated anti-mouse antibodies used were purchased from Biolegend, ThermoScientific, BD Pharmingen, or R&D Systems, and were specific for: Clec 9A (clone 4D2, PE), MHC-I (Kb) (clone AF6-88.5.5.3, APC), DEC205 (clone 205yekta, PerCP-eFluor710), CD24 (clone M1/69, PerCP-Cy5.5), GR1 (clone RB6-8C5, PerCp-Cy5.5), Langerin (clone eBioL31, PE), CD4 (clone RM4-5, APC, PE-Cy7, PE, or eFluor450), CD11b (clone M1/70, APC), Sirpα (clone P84, APC), MHCII (clone M5/114.15.2, PE), CD11c (clone N418, PeCy7), CD8α (clone 53-6.7, APC-Cy7 or PE-Cy7), B220 (clone Ra3-6B2, eFluor450), CD80 (clone 16-10A1, PE), CD40 (clone 1C10, APC), B7-DC (clone Ry25, PE), PD-L1 (clone 1-111A, PE), CD86 (clone GL-1, AlexaFluor700), MHCII (clone M5, PerCp), CD44 (clone IM7, APC, PE-Cy7, or Pacific Blue), CD62L (clone MEL-14, APC-Cy7, FITC or PE), CD25 (clone PC61.5), TIM-3 (clone B8.2C12, PE-Cy7), LAG-3 (clone C9B7W, APC), FoxP3 (clone FJK-16s, PE or PE-Cy5), EBI3 (clone 355022, APC), P35 (clone IC2191P, PE), IL-10 (clone JES5-16E3, FITC or PE). For intracellular staining, cells were re-stimulated with PMA (10 ng/mL) and ionomycin (500 ng/mL) in the presence of Brefeldin A (10 μg/mL) for 4 h. After extracellular staining, cells were fixed with 3.4% formalin for 15 min at RT and permeabilized with 0.5% saponin buffer for 30 min at 4°C or fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher) according to manufacturer's instructions. Cells were analyzed in a LSRII, Canto, or Fortessa flow cytometers (BD), and data processing was done using FACS Diva (BD) and FlowJo (Tree Star). Gates were performed based on Fluorescence Minus One (FMO) controls.

Statistical Analysis

Results were presented as mean values ± SEM. Statistical analysis were determined by the one-way ANOVA, followed by Tukey's multiple comparison test, or two-tailed Student's *t*-test, using GraphPad Prism software (ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

RESULTS

IL-10-Secreting DCs: Altered MHC II and Co-stimulatory Molecule Expression at Steady State and Upon Stimulation

The newly generated IL-10-expressing MutuDC line (referred to as IL10-DC in this work) stably expressed and released IL-10 in resting conditions (**Figures 1A,B**). In order to test if they kept the MutuDC1 phenotype, we used a panel of surface markers to distinguish DC subsets in IL10-DCs and compared them to untransduced (ctrl-DCs) and mock-transduced MutuDCs. The lentiviral transduction did not affect the expression of the distinctive markers for cDC1s: CD11c, CD8α, DEC205, CD24, and Clec9A, and did not lead to the expression of B220 (specific for pDCs), CD11b, CD4, SIRPα (typically expressed by cDC2s), Gr1 (characteristic for monocytes), or langerin (restricted to dermal DCs) (**Supplementary Figure 1**). No important modifications in the immature state of the MutuDC1 line were observed after the viral transduction (data not shown).

Upon stimulation with a combination of the TLR ligands CpG (1 μM) and poly(I:C) (5 μg/mL) for 24 h, IL-10 production by IL10-DCs was approximately five times higher than the production of the cytokine by stimulated ctrl-DCs. Contrary to untransduced DCs, the same augmented amount of IL-10 was detected in IL10-DCs cultured in the absence of the stimuli, suggesting that the cells were already producing the cytokine at maximum rate (**Figure 1B**). In resting conditions, the IL10-DCs expressed lower levels of CD40 and MHC II than control DCs and comparable levels of CD80 and CD86. Upon stimulation with CpG (1 μM) and poly(I:C) (5 μg/mL), IL10-DCs failed to upregulate MHC II expression. CD86 and CD40 were only weakly upregulated upon activation in IL-10 DCs, whereas CD80 expression was only slightly lower in IL-10 DCs than in control DCs (**Figure 1C**).

IL10-DCs Induced T Cell Proliferation but Did Not Enhance Treg, Tr1, or iTr35 Differentiation When Combined With IL35-DCs

We next sought to find out if IL10-DCs were able to induce T cell proliferation and what kind of adaptive immune response could be generated in an antigen-specific manner. IL-10 was shown to impair allogeneic and antigen-specific $CD8^+$ T cell responses (21). To determine whether IL10-DCs could affect $CD8^+$ T cell proliferation, DCs were pulsed with SIINFEKL ($OVA_{257-264}$) for 4 h, washed, and co-cultured with naïve $eFluor^{670}$-labeled OT-I $CD8^+$ T cells for 3 days. IL10-DCs induced similar $CD8^+$ T cell proliferation compared to control DCs (**Supplementary Figure 2A**). The expression of perforin (**Supplementary Figure 2B**) and granzyme B (**Supplementary Figure 2C**) in $CD8^+$ T cells after co-culture was also comparable. Comparable $CD4^+$ T cell proliferation was also observed when IL10-DCs or control DCs were pulsed with OT-II peptide ($OVA_{323-339}$) and co-cultured with $eFluor^{670}$-labeled OT-II $CD4^+$ T cells (**Supplementary Figure 2D**). IL10-DCs were similarly able to process full-length ovalbumin and induce antigen-specific $CD4^+$ T cell proliferation (**Supplementary Figure 2E**).

We have previously thoroughly characterized a high IL-35-producing DC line, also generated from transduced MutuDC1, that expressed low levels of MHC class I and II and failed to upregulate them. Similarly, CD40, CD80, and CD86 was less induced upon stimulation with CpG and poly I:C. When

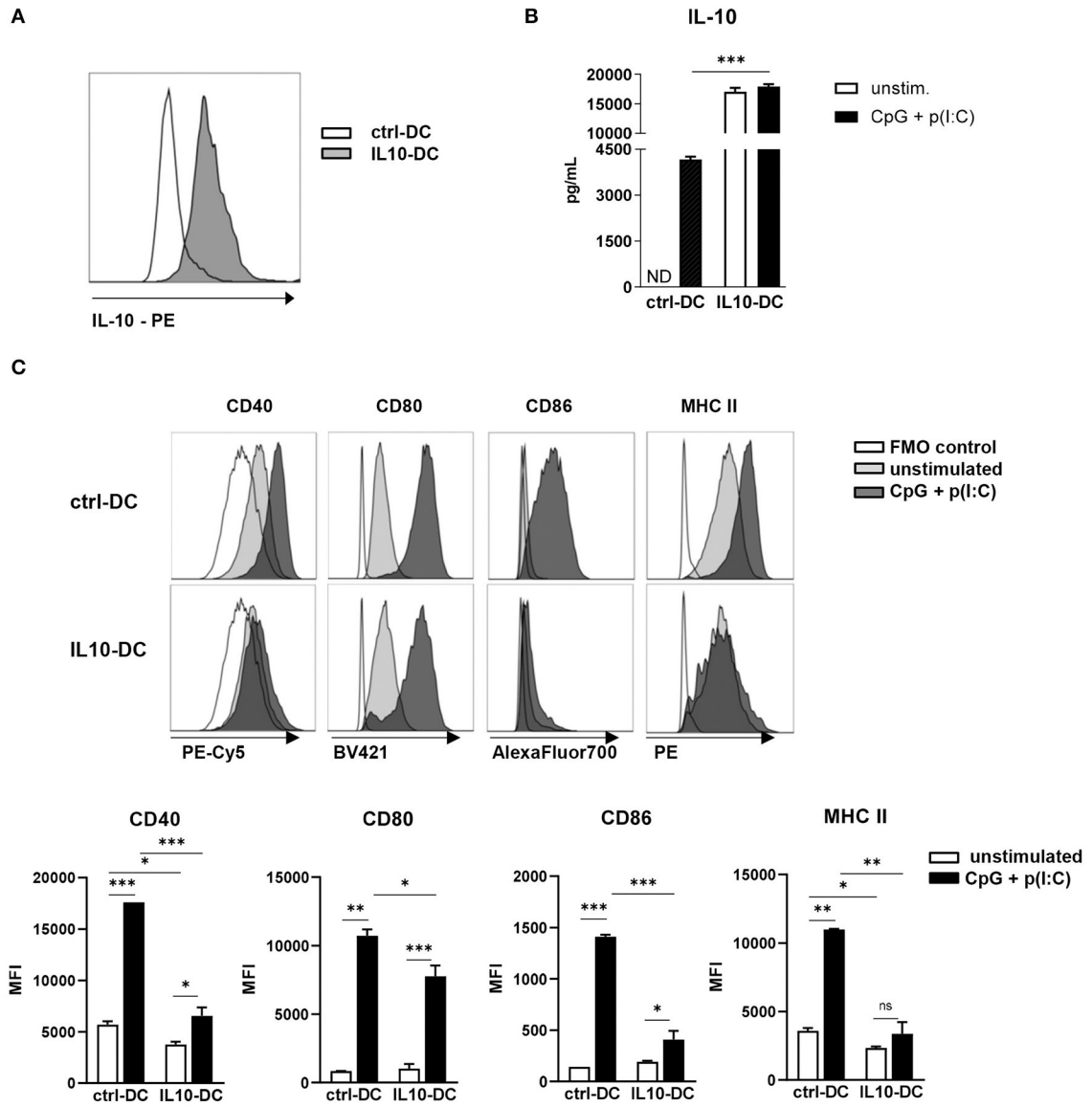


FIGURE 1 | Characterization of the novel IL-10-expressing DC line. 2.5×10^5 control MutuDCs or IL10-DCs or were stimulated with CpG (1 μM) + Poly I:C (5 μg/mL) for 24 h. **(A)** IL-10 expression of control MutuDC (ctrl-DC) and IL10-DC in resting conditions. **(B)** IL-10 secretion assessed in the supernatants of cells cultures. **(C)** Flow cytometric analysis of CD40, CD80, CD86, and MHCII surface expression of ctrl-DC or IL10-DC. Data are expressed in Median Fluorescence Intensity (MFI) of GFP^+-gated cells. Data are representative of at least three independent experiments. ND, not detected. $^*P < 0.05$; $^{**}P < 0.005$, $^{***}P < 0.001$.

compared to mock-transduced MutuDCs, IL35-DCs induced reduced levels of $CD4^+$ and $CD8^+$ T cell proliferation (10). Having observed that IL35-DCs could be efficient in changing immunological memory after Th1/Th17 balances were established, we wondered if in combination with the IL10-DC line their tolerogenic features could be potentiated in a synergistic manner. To do that, we tested the combination of our two DC lines in a T cell proliferation assay. We found that the addition of IL10-DCs in the co-culture failed to restore the impaired $CD4^+$ T cell proliferation induced by the IL35-DCs (**Figure 2A**). Interestingly, CD4 expression in T cells appeared reduced when they had been primed by IL35-DCs (**Figures 2A,B**). The percentages of total induced $CD25^+FoxP3^+$ Tregs were similar, except for a reduced Treg differentiation when the $CD4^+$ T cells were co-cultured with IL10-DCs alone (**Figure 2C**). For Tr1 ($CD4^+$ $IL10^+$ T cell) differentiation, co-culture with IL10-DCs or IL35-DCs alone was significantly more effective than when the cell lines were combined (**Figure 2D**). Surprisingly, the regulatory iTr35 population ($EBI3^+$ $P35^+$ T cells) differentiation was not increased when IL35-DCs were present in the co-cultures (**Figure 2E**). In order to rule out the effect of the cytokine alone, we polyclonally stimulated T cells in the presence

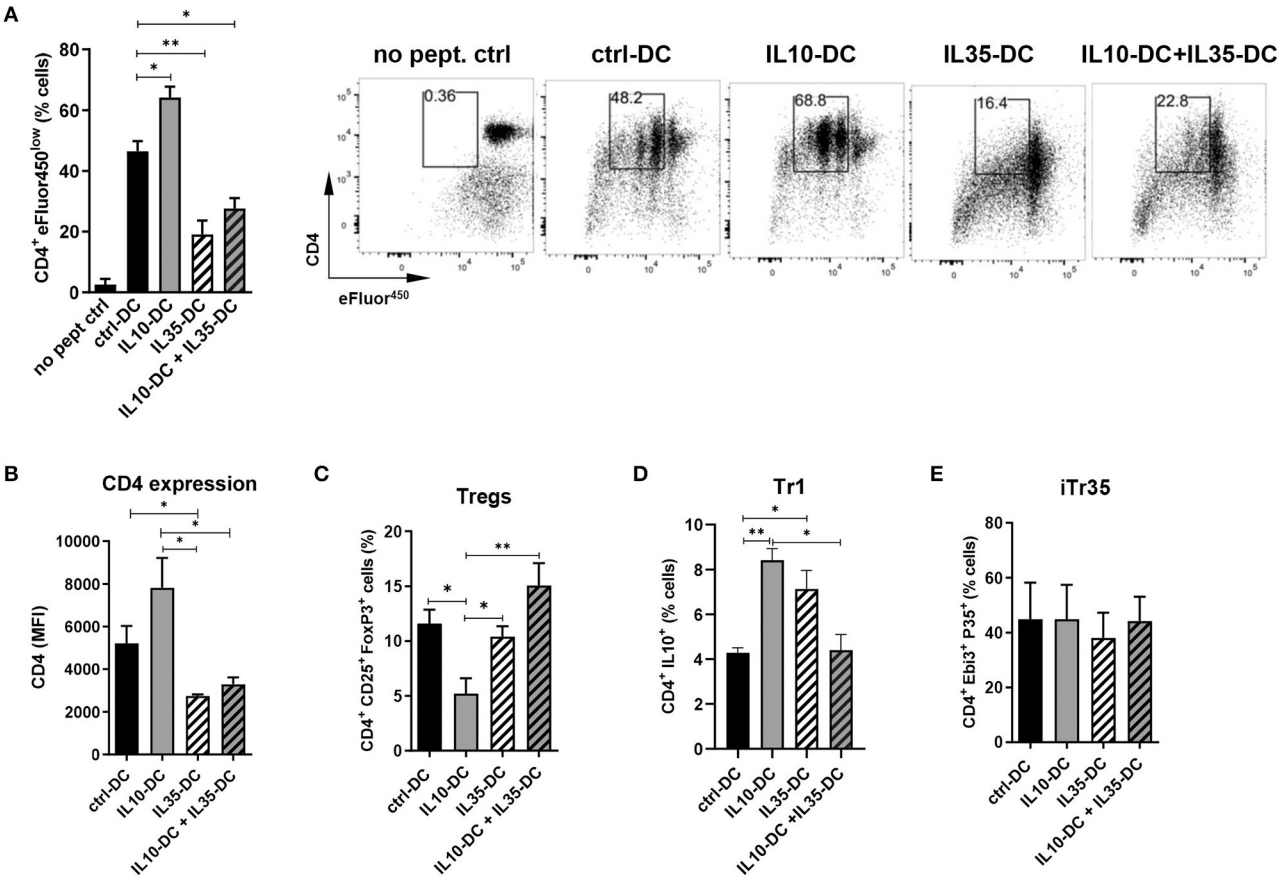


FIGURE 2 | The combination of IL10-DC and IL35-DC did not increase regulatory T cell populations in OVA-specific T cell cultures. 5×10^3 DCs were seeded in 96-well plates and pulsed with $OVA_{323-339}$ peptide for at least 2 h before the addition of 5×10^4 naïve $eFluor^{450}$-labeled OT-II $CD4^+$ T cells. IL10-DC and IL35-DC lines, when added together, were mixed in equal dilutions before the seeding. Flow cytometric analysis of **(A)** cell proliferation, **(B)** CD4 expression (given in Median Fluorescence Intensity, MFI, of GFP^- gated cells), **(C)** Tregs, determined as $CD4^+$ $CD25^+$ $FoxP3^+$ of GFP^-gated cells, **(D)** Tr1, determined as $CD4^+$ $IL10^+$ of GFP^-gated cells, and **(E)** IL35-secreting Tregs (iTr35), determined as $CD4^+$ $EBI3^+$ $P35^+$ of GFP^-gated cells. Data are representative of at least three independent experiments. $^*P < 0.05$; $^{**}P < 0.005$.

of the supernatant of our DCs in parallel. In this setup, no differences in cell proliferation were observed, but EBI3 MFI was found increased when IL35-DC supernatant was added (data not shown), indicating that the impaired proliferation induced by IL35-DCs is dependent on cell-to-cell contact.

TolDCs Reduce Antigen-Specific Inflammation *in vivo*

To observe if the combination of the tolDCs would impact on memory response *in vivo*, we immunized mice with OVA in complete Freund's adjuvant (CFA). Two weeks after the immunization, OVA-pulsed DCs were transferred to immunized mice, which were divided in groups according to the DC line they were going to receive: (1) IL10-DC; (2) IL35-DC; (3) IL10-DC + IL35-DC; (4) ctrl-DC; and finally as a control to see if DCs were boosting the immune response, (5) unpulsed ctrl-DC (**Figure 3A**). One week after DC transfer, mice were challenged with 25 μL of OVA (20 mg/mL in PBS, 500 μg) in the footpad. The same volume of PBS was injected in the contralateral footpad as a control and footpad thicknesses were measured at multiple time-points. After 48 h of challenge, the mice that were injected with IL10-DCs showed a reduced local inflammatory response compared to the ctrl-DC group. This difference was still detected after 56 h, when the footpads of mice from the IL10-DC + IL35-DC group also showed decreased swelling compared to the ctrl-DC and the IL35-DC groups (**Figures 3B,C**). Also, OVA-specific IgG1 and IgG2a antibodies were found decreased in the sera of mice from the IL10-DC + IL35-DC group (**Figure 3D**). We then re-stimulated total cells from the inguinal draining lymph nodes (**Figure 3E**) or spleen (**Figure 3F**) with OVA *ex vivo* and we observed a slight increase of the Breg ($B220^+$ $IL10^+$ cells – lymph nodes) and Tr1 ($CD4^+$ $IL10^+$ – spleen) cell populations in the IL35-DC group compared to the ctrl-DC group. Surprisingly, we did not find any differences in the frequency of Bregs or Tr1 in the IL10-DC group or when the two cell lines were combined. In

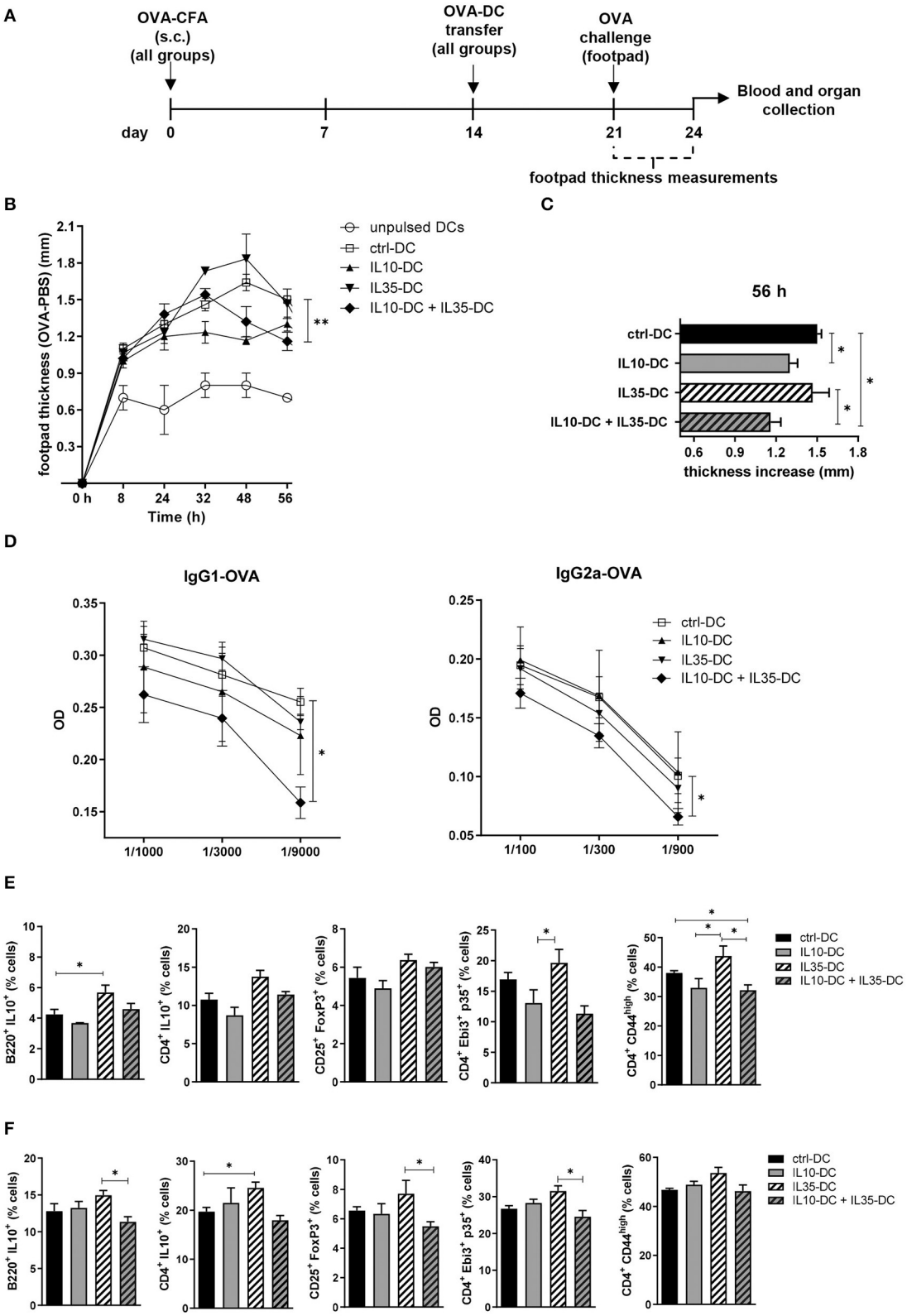


FIGURE 3 | The combination of IL10-DC and IL35-DC ameliorated *in vivo* inflammatory response. Mice were injected s.c. with 50 μg of OVA in Complete Freund's Adjuvant (OVA-CFA) at day 0 and divided in five different groups. At day 14, 3×10^6 OVA-pulsed DCs were injected (via i.p.) and 1 week later they were challenged with a heat-aggregated OVA (20 mg/mL) s.c. injection in the back-right footpad. Same volume of PBS (vehicle) was injected in the contralateral footpad as control.

(Continued)

FIGURE 3 | Footpad thickness was repeatedly measured with a micrometer for 56 h before blood and organ collection. **(A)** Schematic timeline of the experimental procedures. **(B)** Footpad thickness measurements of OVA-injected footpad minus PBS-injected footpads. **(C)** Total increase of OVA-injected footpad thickness after challenge. **(D)** OVA-specific IgG1 and IgG2a antibodies in the serum of mice. Cells from the **(E)** inguinal lymph nodes and **(F)** spleen were re-stimulated with OVA (100 μg/mL) in the presence of brefeldin A (10 μg/mL) overnight and T cell populations were assessed by flow cytometry. Data are representative of at least two independent experiments ($n = 5$ mice/group/experiment). $^{*}P < 0.05$; $^{**}P < 0.005$.

fact, the IL10-DC + IL35-DC group showed reduced numbers in the regulatory cell populations compared to the IL35-DC group, but also decreased numbers of $CD4^{+}CD44^{high}$ cells in the DLNs, indicating impaired T cell activation.

Prevention and Treatment of Established Collagen-Induced Arthritis (CIA)

Rheumatoid arthritis (RA) is an autoimmune disease generally characterized by exacerbated Th1 and Th17 responses, which cause and sustain joint inflammation. Systemic delivery of anti-inflammatory cytokines, such as IL-10 and IL-35 has been shown to prevent the onset of collagen-induced arthritis (CIA) and to treat its symptoms, but the short half-life of some cytokines require constant delivery and high concentrations of the substance, which can cause secondary effects associated with excessive anti-inflammatory response (22, 23). More target-specific approaches employing peptides or cell transfer have also been tested and showed promising results (24). Taking advantage of our tolerogenic cytokine-secreting DC lines, we tested the therapeutic effect of IL-10 and IL-35-secreting DCs in CIA. For that, we immunized the CIA-susceptible $CD11b^{-/-}$ mice with collagen II (CII) in CFA. When over 80% of mice were already showing at least mild scores of the disease (footpad redness and/or swelling of one limb), we transferred a total of 5×10^{6} tolDCs/mouse via i.p. injection. The treatment was repeated 2 days later, and the progression of the disease was then scored for 1 extra week (**Figures 4A,B**). The combination of the two tolDC lines increased the chances of scores being reduced (**Figures 4C,D**). Moreover, in the group of mice treated with the combination of IL10-DCs + IL35-DCs, 75% of mice showed capacity for an intermediate recovery (over 1.5 points in score from the day of the second DC injection to 1 week later), while the groups that were treated with ctrl-DCs, IL10-DCs, or IL35-DCs alone could also reach reduced rates for the same criteria (25, 50, and 33.33%, respectively). Although no significant differences were observed in the linear phase of CII-specific IgG2a or IgG1 antibodies dilution in the sera of mice 1 week after treatment, the group that was injected with IL10-DC + IL35-DCs showed a tendency to produce reduced CII-IgG1 levels, demonstrated by a lower ratio of antibody concentration between the sera of DC-injected mice and of non-injected mice (**Supplementary Figure 3**). In another approach, we tested the systemic delivery of IL-10 in combination or not with IL-35 in the prevention of the disease's onset. For that, mice had a cytokine-secreting implant inserted subcutaneously in their back before the onset of the CIA (**Figure 4E**). Our results showed that the scores were kept lower in mice with IL-10- or IL-35-secreting implants. The systemic delivery of the two cytokines simultaneously, however, did not protect mice from the disease. In the mixed cytokine devices, the ratio of cytokine-producing cells was 1:1, but the total number of cells per device was the same as in the other groups, suggesting that the final concentration of both cytokines was probably suboptimal in the IL10 + IL35 implant group (**Figures 4F,G**). $FoxP3^{+}$ Tregs, Bregs, iTr35, and Tr1 subpopulations in the spleen and inguinal lymph nodes of the mice from the implant groups and the mice that were treated with the DCs were assessed but no differences were found (data not shown). These results indicated that the systemic delivery of either IL-10 or IL-35 alone were significantly effective in the prevention of the disease onset, while the combination of the two cytokines did not show an advantage on it. However, it was only the combination of the IL-10 and IL-35 secreting tolDC lines that was more efficient at reducing the disease scores and promoting a robust recovery in mice with less severe forms of the disease.

The Combination of IL10-DCs and IL35-DCs Did Not Alter Pre-established Memory Through Treg Expansion

As our data indicated that the effect of the tolerogenic cell lines combined was in reducing pre-established memory activation, we generated memory T cells by immunizing mice against OVA (0.5 mg/mL) in CFA to further investigate it. Four weeks after the initial injection, $CD4^{+}$ T cells were extracted from spleens and inguinal lymph nodes, labeled with a proliferation dye and co-cultured with OVA-pulsed tolDCs for 3 days. All DC lines induced comparable $CD4^{+}$ T cell proliferation (**Figure 5A**) but CD4 MFI of total T cells was found reduced after the co-culture with IL35-DCs alone or in combination with IL10-DCs (**Figure 5B**). When central memory cells (T_{CM}, $CD25^{-}$ $CD62L^{+}$ $CD44^{high}$) and effector memory cells (T_{EM}, $CD25^{-}$ $CD62L^{-}$ $CD44^{high}$) compartments were investigated, no differences in the percentage of central and effector memory cells were observed between the experimental groups (**Figures 5C–E**). CD4 expression was downregulated in T cells primed by IL35-DCs alone or in combination with IL10-DCs was found in both, T_{CM} and T_{EM} compartments (**Figures 5F,G**).

The Combination of IL10-DCs and IL35-DCs Induced High LAG-3 Expression in T_{CM}

IL-35 was reported to induce LAG-3, PD-1, and TIM-3 on intratumoral $CD4^{+}$ and $CD8^{+}$ T cells (25). LAG-3 has been recently shown to selectively bind to stable peptide-MHC class II complexes, regulating $CD4^{+}$ T cell activation in an expression-level-dependent fashion (26), therefore we tested if LAG-3 expression was altered by the IL35-DCs and what consequences the association of the IL35-DC and IL10-DC lines would bring to T cell fate. Indeed, LAG-3 expression was upregulated in

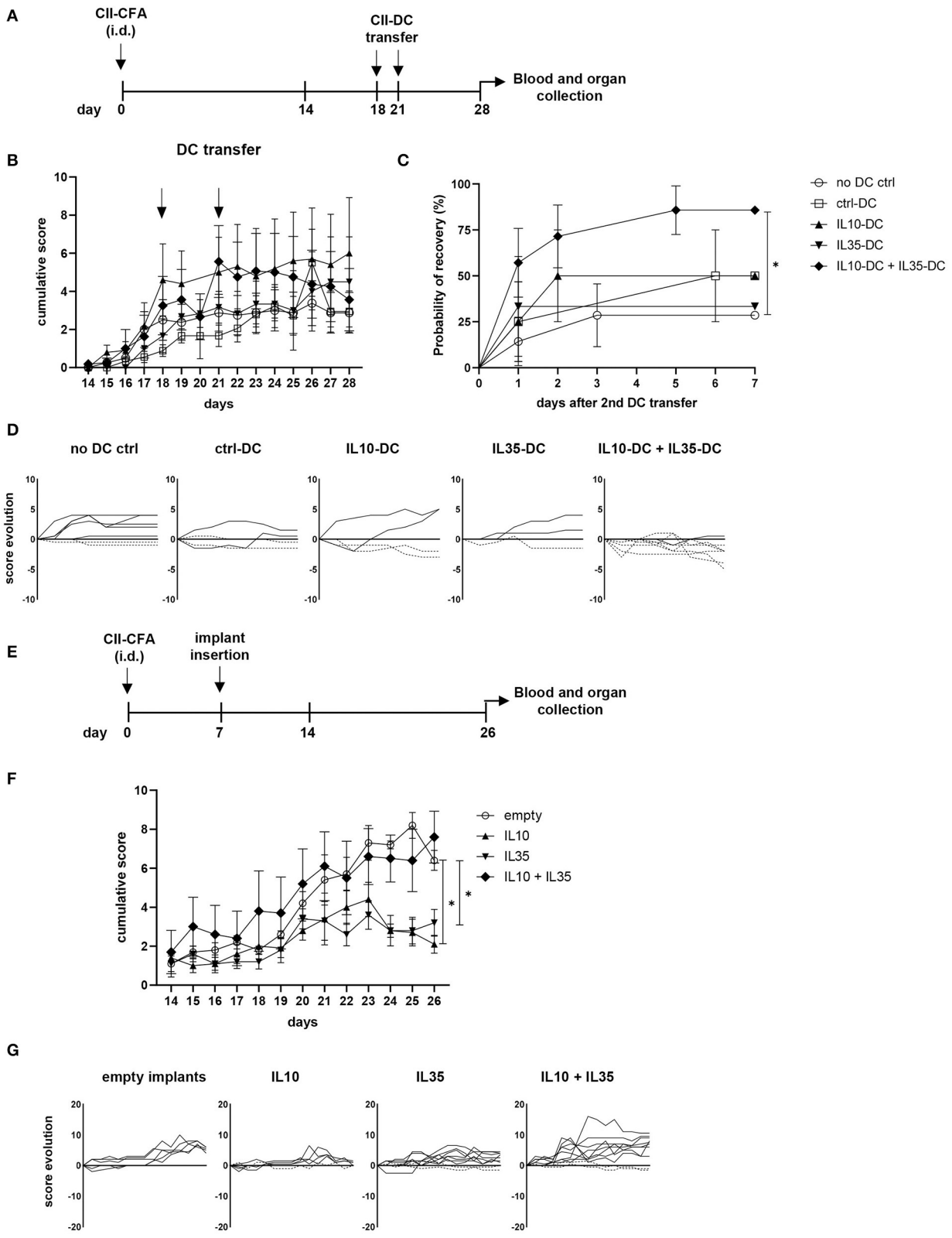


FIGURE 4 | IL10-DC + IL35-DC vaccination reduced established CIA clinical scores. CIA was induced with an i.d. injection at the base of the tail of 100 μg of chicken collagen II emulsified in Complete Freund's Adjuvant (5 mg/mL) (CII-CFA). At day 18, when 80% of mice were showing at least mild scores of the disease, mice were vaccinated with 5×10^6 CII-pulsed DCs (CII-DC). Treatment was repeated 2 days later. Arthritis severity was evaluated daily and each paw was individually scored for

(Continued)

FIGURE 4 | erythema, swelling, and ankylosis. **(A)** Schematic timeline of the experimental procedures. **(B)** Cumulative arthritis scores; CII-DC transfers are indicated by the arrows. **(C)** Probability of recovery evaluation of mice after the second DC transfer based on cumulative score reduction: each mouse was analyzed for having its scores reduced or not. **(D)** Individual score evolution of sick mice after the second DC transfer: each line represents the increase (full lines) or decrease (dashed lines) of the cumulative score (*y* axis) of one individual during days 21 to 28 (*x* axis). In a second approach, 1 week after immunization, five groups of mice were implanted with bioactive cellular implants secreting IL-10, IL-35, IL-10, and IL-35, or empty implants. **(E)** Schematic timeline of the experimental procedures. **(F)** Cumulative arthritis scores. **(G)** Individual score evolution of sick mice 1 week after the implantation of device: each line represents the increase (full lines) or decrease (dashed lines) of the cumulative score (*y* axis) of one individual during days 14 to 26 (*x* axis). Data are representative of at least two independent experiments ($n = 3$–10 mice/group/experiment). $^*P < 0.05$.

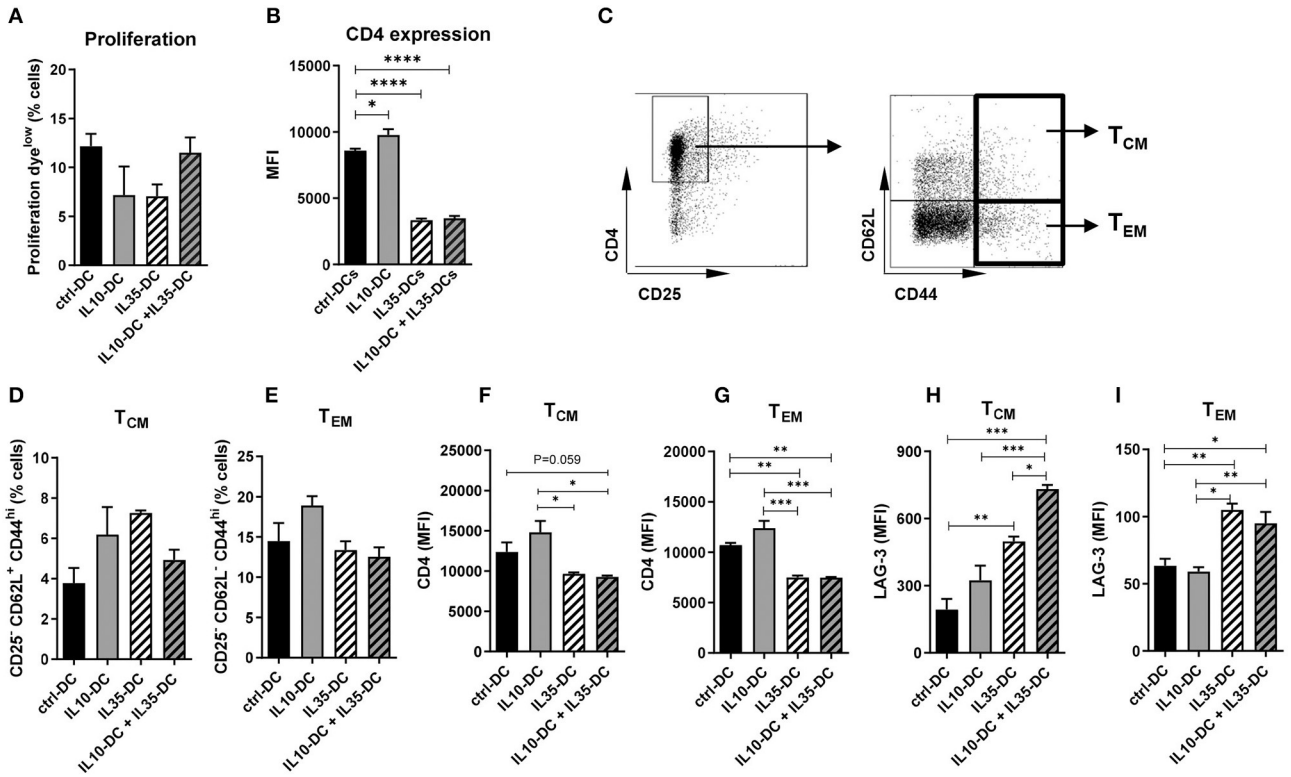


FIGURE 5 | The combination of IL10-DC and IL35-DC induced upregulation of LAG-3 expression on memory CD4$^+$ T cells. CD4$^+$ T cells were isolated from the spleens of OVA-immunized mice, labeled with a proliferation dye, and put in co-culture with OVA-pulsed DCs for 3 days. Flow cytometric analysis of **(A)** T cell proliferation after co-cultures and **(B)** CD4 expression of total cells. **(C)** Gating strategy to determine T central memory (T_{CM}–CD62L$^+$CD44^{high}–**D**) or T effector memory (T_{EM}–CD62L$^-$CD44^{high}–**E**) cells. CD4 expression on **(F)** T_{CM} and **(G)** on T_{EM}, and LAG-3 expression on **(H)** T_{CM} and **(I)** on T_{EM} (given in MFI values). Data are representative of at least three independent experiments with T cells isolated from 3 to 5 independently immunized mice ($n = 3$–5, each experiment). $^*P < 0.05$; $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

both, central and effector memory compartments, when T cells had been co-cultured with IL35-DCs (**Figures 5H,I**). In addition to that, when IL35-DCs were combined with IL10-DCs, the LAG-3 MFI in the T_{CM} compartment was even more increased (**Figure 5H**). We also tested TIM-3 but there were no changes in its expression among the subgroups tested (data not shown).

When the co-cultures were performed in the presence of LAG-3 blocking antibody, CD4$^+$ T cell proliferation induced by ctrl-DCs and by the combination of IL10-DCs plus IL35-DCs was found increased. When T cells were primed by IL35-DCs, on the contrary, proliferation was reduced in a dose-dependent manner (**Figure 6A**). The effect of the blockade of LAG-3 was mostly observed in the reactivated T_{CM} compartment: in the presence of the antibody we observed an expansion of memory T cells after co-cultures with ctrl-DCs or with the combination of IL10-DC + IL35-DCs, and a reduction when co-cultures were done with IL10-DCs or IL35-DCs. In the T_{EM} compartment, the effect observed was the opposite: the blockade of LAG-3 decreased the expansion of the effector memory T cells in the first two groups mentioned above (**Figure 6B**). Altogether these results indicated that the combination of IL10-DCs and IL35-DCs reduced T cell activation through the downregulation of CD4 and upregulation of LAG-3 in the re-activated central memory cells, preventing them to be expanded, thus arresting excessive response during an inflammatory scenario.

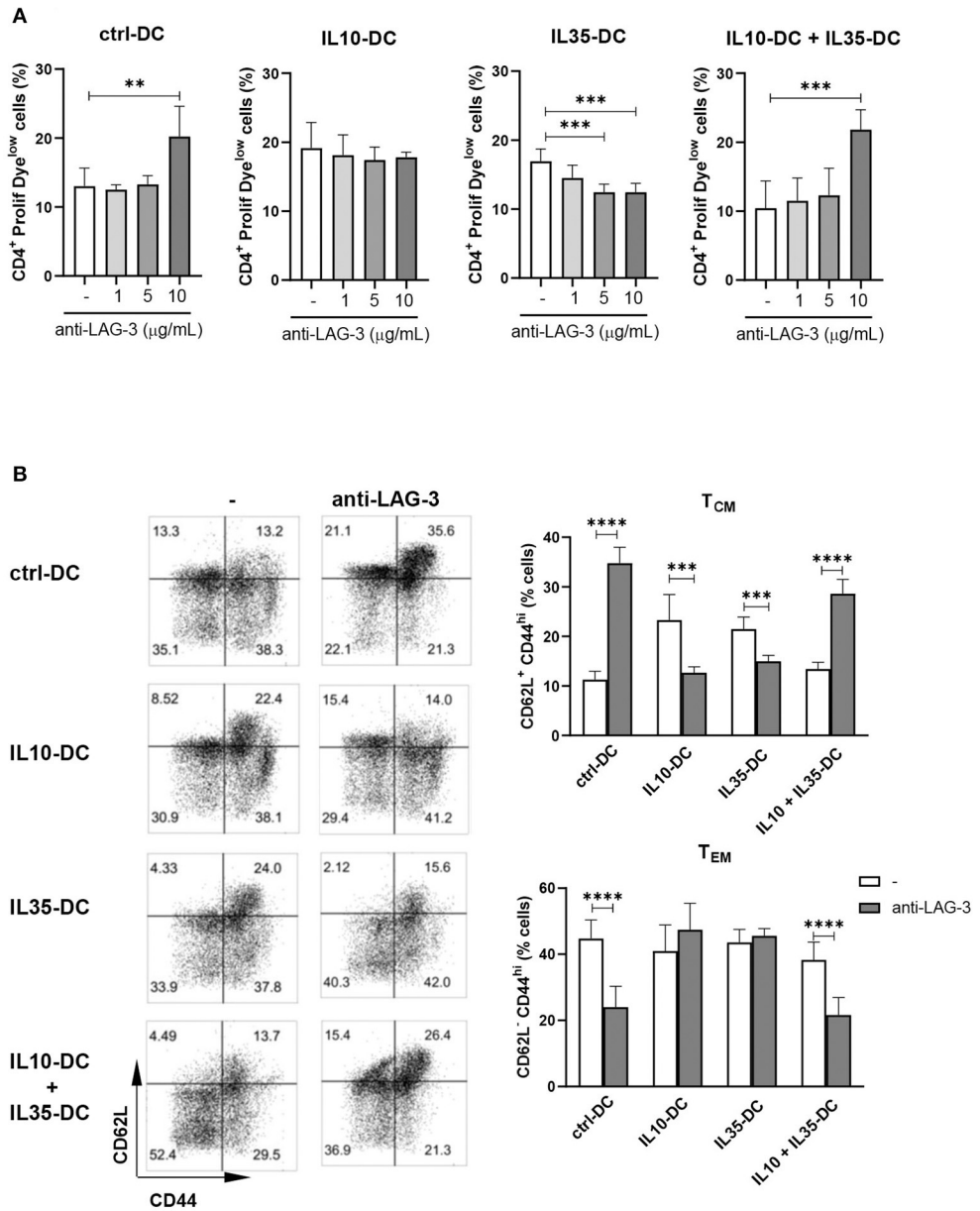


FIGURE 6 | IL10-DCs in combination with IL35-DCs arrest memory expansion through LAG-3. CD4$^+$ T cells were isolated from the spleens of OVA-immunized mice, labeled with a proliferation dye, and put in co-culture with OVA-pulsed DCs in the presence of anti-LAG-3 blocking antibody for 3 day. Flow cytometric analysis of **(A)** T cell proliferation after co-cultures and **(B)** CD4$^+$ memory (CD62L$^+$CD44^{high}) or effector (CD62L$^-$CD44^{high}) cell population. Data are representative of at least two independent experiments with T cells isolated from five independently immunized mice ($n = 5$, each experiment). $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

DISCUSSION

DC vaccination relies on the manipulation of DCs to influence the immune system in an antigen-specific manner toward a tolerogenic or an immunogenic response. The study of DC biology is, however, limited by the scarcity and instability of DCs and thereby, the MutuDC lines represent a great tool to define the benefits of different immunomodulatory molecules in DC-based therapy. For this study, we employed our MutuDCs and generated a new tolerogenic DC line that constitutively produced high levels of IL-10.

TolDCs are characterized by stable low expression of co-stimulatory and MHC molecules, and altered cytokine secretion, leading T cells to anergy or promoting regulatory T cell differentiation. In addition to that, IL-10 is a major player in the anti-inflammatory response, classically known to inhibit T cell responses due to its modulatory effects on APCs or acting directly on the T cells [reviewed by Saraiva et al. (22)]. Moreover, IL-10 is also involved in the differentiation of Tr1

cells, which are able to suppress antigen-specific responses, thus having an important effect on the induction and maintenance of the peripheral tolerance (27). The IL10-DC line displayed many aspects of tolDCs, probably due to the constant and robust exposure to the tolerogenic cytokine secreted. Additionally, our results showed their ability to induce Tr1 differentiation and restrain *in vivo* antigen-specific inflammation. Still, they were able to induce substantial T cell response both *in vitro* and *in vivo*, indicating an advantage over conventional cytokine therapy.

The IL35-DC line was shown to induce suppressive T cells and efficiently reduce EAE scores even after the Th1/Th17 balance was established (10). Our results presented in this study confirmed the tolerogenic properties of the IL-35 secreting MutuDCs. Furthermore, here we show that the combination of IL35-DCs and IL10-DCs promoted a robust recovery of sick mice in an autoimmune model of RA. RA is a chronic, inflammatory, systemic autoimmune disease that can severely damage the joints and impair life quality. There is no cure for RA and the progression of the disease can lead to irreversible disability. Even though it is generally difficult to cure an established autoimmunity, different treatments for RA that allow many to live a near-normal life are available. In fact, most of the drugs currently used to treat RA are anti-inflammatory immune modulators, like Infliximab (anti-TNFα), Abatacept (CTLA-4Ig), Tocilizumab (anti-IL6R), Rituximab (anti-CD20), Anakinra (IL1-R antagonist), Canakinumab (anti-IL1), and Tofacitinib (JAK inhibitor). Some of these approaches are also proposed to treat other autoimmune diseases, such as Crohn's disease, ulcerative colitis, psoriasis, and have been shown to help in most cases, but the constant need of monitoring the disease and adjusting the treatment accordingly makes it challenging to achieve clinical remission (28, 29). On top of this, those broad spectra acting drugs, present a strong impact on the host defense as they modulate inflammatory and immune mediators, creating an eminent risk of infection. Thus, targeting the pathogenic autoreactive cells and/or antibodies in an antigen-specific fashion can exempt the protective immune cells and healthy tissues from collateral damage. While peptide-MHC-specific monoclonal antibodies have not yet been approved for therapeutic use, tolDC-based immunotherapeutic approaches have been conducted in the past decades aiming to induce, enhance, or restore tolerance in an antigen-specific fashion. In fact, many clinical trials with DCs differentiated in the presence of tolerogenic factors, such as IL-10, vitamin D, NF-κB inhibitor, have been reported with positive results, but the establishment of a standard DC manufacturing protocol, their tolerogenic characteristics stability, mode of action, and so on still remain elusive (7). Cytokine therapy can also represent an alternative to treat autoimmunity. The administration of IL-10, for example, has already shown promising results in the treatment of RA (30) and psoriasis (31). Moreover, the association of IL-10 with other anti-inflammatory cytokines could also be advantageous. The cooperation of IL-10 and TGF-β in downregulating immune responses is widely known as their production and action are interrelated. Additionally, the two cytokines were shown to cooperate in inducing secondary hyporesponsiveness to alloantigen (32), generating potent regulatory cells (12), and even inhibiting humoral immune response in a synergistic fashion (33), which suggests that combinatory cytokine therapies could increase the efficacy of the treatment without the possible side effects caused by high doses of single mediators. Thus, our results with systemic delivery of anti-inflammatory cytokines also provide new insights on therapeutic strategies for systemic inflammatory diseases.

In the immunological synapse, CD4 acts as a co-receptor for MHC class II, contributing to the assembly of the TCR-MHC II complexes. CD4 expression was reported to be upregulated after TCR triggering, which in turn correlates with increased T cell proliferation (34). On the other hand, low expression of CD4 could decrease T cell sensitivity to antigens and the efficiency of TCR-peptide-MHC II (pMHCII) binding. Through the blockade of IL-35 or Treg-restricted deletion of IL-35, Turnis et al. showed that IL-35 was implicated in promoting the expression of the inhibitory receptors PD-1, TIM-3, and LAG-3 in $CD8^+$ tumor infiltrating T cells (25). LAG-3 structurally resembles CD4 but binds to MHC class II with a higher affinity than CD4, acting as a regulator of the immune response. This receptor has been also reported to be expressed in $FoxP3^+$ Tregs and Tr1 cells, but most of all, its expression correlates with high IL-10 secretion [reviewed by Anderson et al. (35)]. Using multiple *in vivo* systems to induce immune responses in LAG-$3^{-/-}$ mice, Workman et al. showed that LAG-3 negatively regulated primary T cell expansion and memory development (36). Among memory T cells, the T_{CM} cells comprise a population of lymph node-homing and circulatory cells that have a greater capacity of proliferation upon reactivation; they have less co-stimulation dependency and have a lower activation threshold, thus they are more likely to become activated during a second encounter with the antigen and providing stronger and faster responses (37). The distinct effect observed on T_{CM} expansion in the presence of anti-LAG-3 blocking antibody indicated that the two cytokine-secreting cell lines work differently in the induction of tolerance, and that when combined, the effect observed is most likely the result of a cooperation between them. As shown by Maruhashi et al. (26), the inhibition of $CD4^+$ T cell activation through LAG-3 is dependent on stable pMHCII recognition. Due to the impaired T cell priming activity of IL35-DCs, we find unlikely that stable pMHCII complexes were formed by these cells. IL10-DCs could, however, provide those stable complexes, thus preferentially leading to their binding to LAG-3 instead of CD4. Thus, we hypothesize that when the two cell lines were combined, LAG-3 expression in T cells was induced and potentiated by the combination of the two cytokines secreted, but the T cell-DC binding mainly occurred with IL10-DCs. This could explain the enhanced upregulation of LAG-3, but similar proliferation induced by the two tolDC lines combined. In this scenario, IL35-DCs alone would induce tolerance through a different mechanism other than LAG-3 expression, and T cell priming by IL10-DCs alone would not be affected by the blockade of LAG-3. Nevertheless, the decreased proliferation induced by IL35-DCs under anti-LAG3 blockade still requires further studies to investigate if IL-35 could prompt an inverse agonist activity of the blocking antibody in memory T cells.

Taken together, our results demonstrate that MutuDC lines represent a great tool to investigate the benefits of immunomodulatory molecules in the antigen-presentation context and thus could help to characterize and optimize potential treatments for autoimmune disorders where overly inflammatory conditions are established.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Cantonal Veterinary Office of Vaud, Switzerland.

AUTHOR CONTRIBUTIONS

MK, AE, MP, CL, MS, and VL conducted the experiments. MK, BS, and HA-O designed the experiments. MK wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This project was funded by the Swiss National Science Foundation grant number 310030_170083 to HA-O.

ACKNOWLEDGMENTS

The authors thank Dr. Anaïs Duval for starting this project, Dr. Sergio Haller and Dr. Sunil Raghav for their availability to discuss it, Vanessa Mack for technical assistance, and the animal caretakers of the animal facility at the CIIL for their support in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.607315/full#supplementary-material

Supplementary Figure 1 | Phenotypical characteristics of the IL10-DC line after transduction. Cells were analyzed for the indicated surface markers by Flow cytometry after lentiviral transduction. Data are representative of three independent experiments.

Supplementary Figure 2 | IL10-DC induce antigen-specific T cell proliferation. 5×10^3 transduced or untransduced DCs were seeded in 96-well plates and pulsed with $OVA_{257-264}$ peptide (SIINFEKL), $OVA_{323-339}$ peptide (OT-II peptide), or full-length ovalbumin (100 μg/mL) for at least 2 h before adding 5×10^4 of naïve proliferation dye-labeled OT-I $CD8^+$ or OT-II $CD4^+$ T cells. Cells were left in co-culture with peptide-pulsed DCs for 3 days or with OVA-pulsed DCs for 4 days. Flow cytometric analysis of **(A)** $CD8^+$ T cell proliferation, **(B)** perforin, and **(C)** granzyme B expression on $CD8^+$ T cells; **(D,E)** $CD4^+$ T cell proliferation. Data are representative of at least three independent experiments. Bar graphs indicate the mean of technical replicates in one representative experiment.

Supplementary Figure 3 | The combination of IL10-DC and IL35-DC lines reduced the IgG1-CII antibody levels in CIA sick mice. Blood serum samples from sick mice were obtained after 28 days of CIA induction. **(A)** Collagen II-specific IgG1 and IgG2a antibodies. **(B)** Ratio between the average concentration of CII-specific antibodies in DC-injected mice and non-injected mice. Data are representative of two independent experiments (n = 3–8 mice/group/experiment).

REFERENCES

1. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 392:245–52. doi: 10.1038/32588
2. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood*. (2002) 99:2468–76. doi: 10.1182/blood.v99.7.2468
3. Torres-Aguilar H, Aguilar-Ruiz SR, González-Pérez G, Munguía R, Bajaña S, Meraz-Ríos MA, et al. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory $CD4^+$ T cells. *J Immunol*. (2010) 184:1765–75. doi: 10.4049/jimmunol.0902133
4. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*. (2005) 6:1219–27. doi: 10.1038/ni1265
5. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science*. (2018) 359:1350–5. doi: 10.1126/science.aar4060
6. Boks MA, Kager-Groenland JR, Haasjes MS, Zwaginga JJ, van Ham SM, ten Brinke A. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction–a comparative study of human clinical-applicable DC. *Clin Immunol*. (2012) 142:332–42. doi: 10.1016/j.clim.2011.11.011
7. Domogalla MP, Rostan PV, Raker VK, Steinbrink K. Tolerance through education: how tolerogenic dendritic cells shape immunity. *Front Immunol*. (2017) 8:1764. doi: 10.3389/fimmu.2017.01764
8. Fuertes Marraco SA, Grosjean F, Duval A, Rosa M, Lavanchy C, Ashok D, et al. Novel murine dendritic cell lines: a powerful auxiliary tool for dendritic cell research. *Front Immunol*. (2012) 3:331. doi: 10.3389/fimmu.2012.00331
9. Pigni M, Ashok D, Acha-Orbea H. Derivation and utilization of functional CD8(+) dendritic cell lines. *Methods Mol Biol*. (2016) 1423:39–49. doi: 10.1007/978-1-4939-3606-9_3
10. Haller S, Duval A, Migliorini R, Stevanin M, Mack V, Acha-Orbea H. Interleukin-35-producing CD8α(+) dendritic cells acquire a tolerogenic state and regulate T cell function. *Front Immunol*. (2017) 8:98. doi: 10.3389/fimmu.2017.00098
11. O'Garra A, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C. Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev*. (2008) 223:114–31. doi: 10.1111/j.1600-065X.2008.00635.x
12. Chen ZM, O'Shaughnessy MJ, Gramaglia I, Panoskaltsis-Mortari A, Murphy WJ, Narula S, et al. IL-10 and TGF-beta induce alloreactive $CD4^+CD25^-$ T cells to acquire regulatory cell function. *Blood*. (2003) 101:5076–83. doi: 10.1182/blood-2002-09-2798
13. Sawant DV, Yano H, Chikina M, Zhang Q, Liao M, Liu C, et al. Adaptive plasticity of IL-10+ and IL-35+ Treg cells cooperatively promotes tumor T cell exhaustion. *Nat Immunol*. (2019) 20:724–35. doi: 10.1038/s41590-019-0346-9
14. Okazaki T, Okazaki IM, Wang J, Sugiura D, Nakaki F, Yoshida T, et al. PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. *J Exp Med*. (2011) 208:395–407. doi: 10.1084/jem.20100466
15. Bettini M, Szymczak-Workman AL, Forbes K, Castellaw AH, Selby M, Pan X, et al. Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. *J Immunol*. (2011) 187:3493–8. doi: 10.4049/jimmunol.1100714

16. Zhang Q, Chikina M, Szymczak-Workman AL, Horne W, Kolls JK, Vignali KM, et al. LAG3 limits regulatory T cell proliferation and function in autoimmune diabetes. *Sci Immunol*. (2017) 2:eaah4569. doi: 10.1126/sciimmunol.aah4569
17. Kadowaki A, Miyake S, Saga R, Chiba A, Mochizuki H, Yamamura T. Gut environment-induced intraepithelial autoreactive CD4(+) T cells suppress central nervous system autoimmunity via LAG-3. *Nat Commun*. (2016) 7:11639. doi: 10.1038/ncomms11639
18. Duval A, Fuertes Marraco SA, Schwitter D, Leuenberger L, Acha-Orbea H. Large T antigen-specific cytotoxic T cells protect against dendritic cell tumors through perforin-mediated mechanisms independent of CD4 T cell help. *Front Immunol*. (2014) 5:338. doi: 10.3389/fimmu.2014.00338
19. Lathuilière A, Cosson S, Lutolf MP, Schneider BL, Aebischer P. A high-capacity cell macroencapsulation system supporting the long-term survival of genetically engineered allogeneic cells. *Biomaterials*. (2014) 35:779–91. doi: 10.1016/j.biomaterials.2013.09.071
20. Stevanin M, Busso N, Chobaz V, Pigni M, Ghassem-Zadeh S, Zhang L, et al. CD11b regulates the Treg/Th17 balance in murine arthritis via IL-6. *Eur J Immunol*. (2017) 47:637–45. doi: 10.1002/eji.201646565
21. Steinbrink K, Jonuleit H, Müller G, Schuler G, Knop J, Enk AH. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. *Blood*. (1999) 93:1634–42.
22. Saraiva M, Vieira P, O'Garra A. Biology and therapeutic potential of interleukin-10. *J Exp Med*. (2020) 217:e20190418. doi: 10.1084/jem.20190418
23. Kochetkova A, Golden S, Holderness K, Callis G, Pascual DW. IL-35 stimulation of $CD39^+$ regulatory T cells confers protection against collagen II-induced arthritis via the production of IL-10. *J Immunol*. (2010) 184:7144–53. doi: 10.4049/jimmunol.0902739
24. Serra P, Santamaria P. Antigen-specific therapeutic approaches for autoimmunity. *Nat Biotechnol*. (2019) 37:238–51. doi: 10.1038/s41587-019-0015-4
25. Turnis ME, Sawant DV, Szymczak-Workman AL, Andrews LP, Delgoffe GM, Yano H, et al. Interleukin-35 limits anti-tumor immunity. *Immunity*. (2016) 44:316–29. doi: 10.1016/j.immuni.2016.01.013
26. Maruhashi T, Okazaki I, Sugiura D, Takahashi S, Maeda TK, Shimizu K, et al. LAG-3 inhibits the activation of CD4. *Nat Immunol*. (2018) 19:1415–26. doi: 10.1038/s41590-018-0217-9
27. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. (1997) 389:737–42. doi: 10.1038/39614
28. Feldmann M, Maini RN. Perspectives from masters in rheumatology and autoimmunity: can we get closer to a cure for rheumatoid arthritis? *Arthritis Rheumatol*. (2015) 67:2283–91. doi: 10.1002/art.39269
29. Hosseini A, Gharibi T, Marofi F, Javadian M, Babaloo Z, Baradaran B. Janus kinase inhibitors: a therapeutic strategy for cancer and autoimmune diseases. *J Cell Physiol*. (2020) 235:5903–24. doi: 10.1002/jcp.29593
30. Trachsel E, Bootz F, Silacci M, Kaspar M, Kosmehl H, Neri D. Antibody-mediated delivery of IL-10 inhibits the progression of established collagen-induced arthritis. *Arthritis Res Ther*. (2007) 9:R9. doi: 10.1186/ar2115
31. McInnes B, Illei GG, Danning CL, Yarboro CH, Crane M, Kuroiwa T, et al. IL-10 improves skin disease and modulates endothelial activation and leukocyte effector function in patients with psoriatic arthritis. *J Immunol*. (2001) 167:4075–82. doi: 10.4049/jimmunol.167.7.4075
32. Zeller JC, Panoskaltsis-Mortari A, Murphy WJ, Ruscetti FW, Narula S, Roncarolo MG, et al. Induction of $CD4^+$ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. *J Immunol*. (1999) 163:3684–91.
33. Komai T, Inoue M, Okamura T, Morita K, Iwasaki Y, Sumitomo S, et al. Transforming growth factor-β and interleukin-10 synergistically regulate humoral immunity. *Front Immunol*. (2018) 9:1364. doi: 10.3389/fimmu.2018.01364
34. Wiegers GJ, Stec IE, Klinkert WE, Reul JM. Glucocorticoids regulate TCR-induced elevation of CD4: functional implications. *J Immunol*. (2000) 164:6213–20. doi: 10.4049/jimmunol.164.12.6213
35. Anderson C, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity*. (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
36. Workman J, Cauley LS, Kim IJ, Blackman MA, Woodland DL, Vignali DA. Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation *in vivo*. *J Immunol*. (2004) 172:5450–5. doi: 10.4049/jimmunol.172.9.5450
37. Raphael A, Joern RR, Forsthuber TG. Memory CD4. *Cells*. (2020) 9:531. doi: 10.3390/cells9030531

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 17 February 2021
doi: 10.3389/fimmu.2021.621039

A Novel Inflammatory Dendritic Cell That Is Abundant and Contiguous to T Cells in the Kidneys of Patients With Lupus Nephritis

*Samir V. Parikh[1], Ana Malvar[2], John Shapiro[1], James M. Turman[1], Huijuan Song[1], Valeria Alberton[3], Bruno Lococo[2], Juan M. Mejia-Vilet[4], Sethu Madhavan[1], Jianying Zhang[5], Lianbo Yu[5], Anjali A. Satoskar[1], Dan Birmingham[1], Wael N. Jarjour[6], Brad H. Rovin[1] and Latha P. Ganesan[1,6]**

[1] Division of Nephrology, The Ohio State University Wexner Medical Center, Columbus, OH, United States, [2] Nephrology Unit, Hospital Fernandez, Buenos Aires, Argentina, [3] Department of Pathology, Hospital Fernandez, Buenos Aires, Argentina, [4] Department of Nephrology and Mineral Metabolism, Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, Mexico City, Mexico, [5] Department of Biomedical Informatics, The Ohio State University, Columbus, OH, United States, [6] Division of Rheumatology and Immunology, The Ohio State University Wexner Medical Center, Columbus, OH, United States

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Sun Jung Kim,
Northwell Health, United States
Carlo Chizzolini,
Université de Genève, Switzerland

***Correspondence:**
Latha P. Ganesan
Latha.Ganesan@osumc.edu

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: 24 October 2020
Accepted: 25 January 2021
Published: 17 February 2021

Citation:
Parikh SV, Malvar A, Shapiro J, Turman JM, Song H, Alberton V, Lococo B, Mejia-Vilet JM, Madhavan S, Zhang J, Yu L, Satoskar AA, Birmingham D, Jarjour WN, Rovin BH and Ganesan LP (2021) A Novel Inflammatory Dendritic Cell That Is Abundant and Contiguous to T Cells in the Kidneys of Patients With Lupus Nephritis. Front. Immunol. 12:621039. doi: 10.3389/fimmu.2021.621039

The mechanisms that promote local inflammatory injury during lupus nephritis (LN) flare are largely unknown. Understanding the key immune cells that drive intrarenal inflammation will advance our knowledge of disease pathogenesis and inform the development of new therapeutics for LN management. In this study, we analyzed kidney biopsies from patients with proliferative LN and identified a novel inflammatory dendritic cell (infDC) population that is highly expressed in the LN kidney, but minimally present in healthy human kidneys. During an agnostic evaluation of immune transcript expression in the kidneys of patients with proliferative LN, the most abundantly overexpressed transcript from isolated glomeruli was *FCER1G,* which encodes the Fc receptor gamma chain (FcRγ). To identify the cell types expressing FcRγ that infiltrate the kidney in LN, studies were done on kidney biopsies from patients with active LN using confocal immunofluorescence (IF) microscopy. This showed that FcRγ is abundantly present in the periglomerular (PG) region of the kidney and to a lesser extent in the tubulointerstitium (TI). Further investigation of the surface markers of these cells showed that they were FcRγ$^+$, MHC II$^+$, CD11c$^+$, CD163$^+$, CD5$^-$, DC-SIGN$^+$, CD64$^+$, CD14$^+$, CD16$^+$, SIRPα$^+$, CD206$^-$, CD68$^-$, CD123$^-$, CD3$^-$, and CD11b$^-$, suggesting the cells were infDCs. Quantification of the infDCs showed an average 10-fold higher level of infDCs in the LN kidney compared to the healthy kidneys. Importantly, IF identified CD3$^+$ T cells to be adjacent to these infDCs in the PG space of the LN kidney, whereas both cell types are minimally present in the healthy kidney. Thus, we have identified a previously undescribed DC in lupus kidneys that may interact with intrarenal T cells and play a role in the pathogenesis of kidney injury during LN flare.

Keywords: inflammatory dendritic cells, lupus nephritis, kidney, autoimmunity, adaptive immune response, T cells, SLE

INTRODUCTION

Lupus nephritis (LN) is a severe complication of systemic lupus erythematosus (SLE) that is associated with considerable morbidity and mortality. Up to 30% of patients with LN progress to end-stage kidney disease (ESKD) (1). There are no specific United States Food and Drug Administration (FDA) approved therapies to treat LN, and the current therapies produce suboptimal response rates with considerable cytotoxicity (2). We and other investigators have been exploring the molecular pathology of the kidney during active LN to better understand the pathogenesis of kidney injury in LN and pathways that may be specifically targeted to treat LN.

During the course of an agnostic evaluation of transcript expression in laser microdissected kidney tissue from clinical LN biopsies, we found that the most abundantly overexpressed transcript in the glomerular compartment was *FCER1G,* encoding the Fc receptor gamma chain (FcRγ). It was assumed that this transcript reflected immune cells infiltrating the kidney during active LN, and this work was undertaken to identify the cell types represented by the overexpressed FcRγ. Thus, in this study, transcriptomic findings were used to guide confocal immunofluorescence (IF) studies to characterize the major infiltrating immune cells present in the kidney during LN flare. We identified a unique population of FcRγ-expressing inflammatory dendritic cell (infDC) that resides in the periglomerular (PG) space and adjacent to $CD3^+$ T cells signifying a potential cross-talk between infDC and T cell populations.

MATERIALS AND METHODS

Experimental Design

The purpose of this work was to perform transcriptional analysis and IF on kidney biopsies done at the LN flare to identify the major infiltrating immune cells present in the kidney at the time of the LN flare. Transcriptomic analysis was performed on kidney biopsies obtained at the LN flare from 58 patients with proliferative (Class III/IV ± V) LN between 2007 and 2013. Archival biopsies were used after clinical testing was completed. Laser capture microdissection (LCM) was performed, and glomeruli and tubulointerstitium (TI) were isolated separately. Preimplantation living donor kidney biopsies ($n = 10$) served as healthy controls (HCs) and were analyzed in parallel with LN biopsies. The same nephrologist (A.M.) treated all the patients, and one experienced nephropathologist (V.A.) read kidney biopsies. Hospital Fernandez (Buenos Aires) ethics board and The Ohio State University institutional review board approved the investigation of the kidney biopsies.

RNA Extraction and Analysis

The biopsies used for transcriptomic analysis were fixed in formalin and paraffin-embedded (FFPE). From the paraffin blocks, 10 μm sections were cut from each biopsy. After deparaffinization, all available glomeruli and TI were separated by laser microdissection (PALM MicroBeam, Zeiss Labs, Bernried, Germany), captured, and digested with proteinase K. DNA was removed with DNase. RNA was precipitated, extracted with RNeasy MinElute spin columns (Qiagen, Redwood City, CA, USA), and eluted in RNase-free water. Transcript expression was analyzed from 250 ng of extracted RNA using the NanoString nCounter platform and the GX human immunology transcript panel [NanoString Technologies, Seattle, WA, USA; (3–5)]. The human immunology panel v2 consisted of 579 immune response genes, 6 positive control genes, and 6 negative control genes. A complete list of these genes can be found in the earlier publication from our group (6).

For confocal IF microscopy, frozen kidney biopsy tissues from four patients with active Class IV LN were obtained from the Ohio State Nephropathology Biorepository. Three frozen nephrectomy samples were used as HC. The nephrectomies were performed in patients with renal cell carcinoma. Tissue obtained for analysis was sectioned away from the cancer tissue. The surrounding tissue used for analysis appeared healthy by histologic analysis. Nephrectomies were used as controls because frozen samples were needed, and we did not have frozen transplant donor tissue stored in our biorepository.

Antibodies

The primary antibodies (Abs) used for IF are all listed in **Supplementary Table 1**. The antibodies used in this study were validated for IF by either using human lymph node or using human liver as positive control (data not shown). The isotype controls used are ChromoPure normal rabbit IgG, normal mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA), mouse IgG1κ (BioLegend, San Diego, CA, USA), and mouse IgG2bκ (Jackson ImmunoResearch). The secondary antibodies used for IF were goat F(ab′)2 anti-mouse IgG 488 (Jackson ImmunoResearch) and goat anti-rabbit IgG 568, goat anti-rabbit IgG 488, and goat anti-rabbit IgG 647 from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence

Frozen nephrectomy and LN kidney biopsies were sectioned (5 μm section per slide), fixed in 4% paraformaldehyde-phosphate buffered saline (PBS) for 15 min at room temperature, and washed with PBS (with 0.02% sodium azide). The sections were blocked with 5% milk in PBS, followed by incubation with the primary Ab overnight. After three washes with PBS for 1 h, the sections were incubated with fluorescently tagged secondary Abs for another hour at room temperature, and nuclei were stained with DAPI (100 ng/ml) for 10 min. The sections were then mounted with Prolong Gold (Invitrogen) under coverslips. Control Abs refer to the list of isotype Abs with their respective secondary Ab. The images were obtained using an Olympus FluoView 1000 Laser Scanning Confocal microscope equipped (Olympus Corp., Tokyo, Japan) with a spectral detection system for a finer separation of fluorochromes (FV1000 spectra) along with ×60 oil immersion lens at room temperature.

Quantitative Microscopy

The expression level of infDC in LN and HC kidneys was quantified from images that were stained for infDC using anti-CD163. The total intensity of CD163 based on the infDC expression was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). CD163 intensity was obtained after subtracting the background fluorescence from the isotype plus secondary Ab-stained images and by measuring the area and the mean fluorescence intensity of the green pixels emanating from infDC using the CD163 antibody as described earlier (7).

Statistical Analysis

For transcriptomic analysis, descriptive statistics are presented as mean ± standard deviation or as a percentage. For clinical variables, *t*-tests, ANOVA, or Wilcoxon rank sum tests were applied as appropriate. For categorical clinical variables, Fisher's exact test was used. For nanostring data, raw counts were normalized to the positive spike-in controls and then log2 transformed. To reduce technical bias, the genes with an expression level below the mean plus two standard deviations of the negative controls were filtered out. Quantile normalization was applied to the remaining transcripts (522 for glomeruli and 502 for TI). Glomerular samples were analyzed separately from TI samples. To determine differential expression, glomerular and TI transcript expression from the LN kidneys were compared to the controls. A 1.5-fold change and $p < 0.05$ were necessary for a transcript to be considered differentially expressed.

For statistical analysis of confocal microscopy, a two-tailed Student's *t*-test was used for two-group comparison, and $p < 0.05$ was considered significant. All analyses were run using Origin Pro version 2020 (OriginLab Corp., Northampton, MA, USA).

RESULTS

Transcriptomic Analysis of Kidney Biopsies at LN Flare Reveals Significant Overexpression of *FCER1G* in the Glomeruli and TI at LN Flare

We performed transcriptomic analysis on RNA isolated from glomeruli and TI using LCM from the kidney biopsies obtained at proliferative LN flare ($n = 58$). Preimplantation living donor kidney transplant biopsies were used as HCs ($n = 10$). Transcriptomic analysis for 579 immune transcripts showed *FCER1G* to be the most significantly overexpressed glomerular transcript [Fold Change (FC): 3.6, *p*-value (*p*): 1E-10] but was also significantly overexpressed in the TI (FC: 1.7, $p = 0.001$) at the LN flare (**Figure 1** and **Supplementary Table 2**) compared to HC. Additionally, the *FCER1G* expression correlated with the NIH histologic disease activity index ($r = -0.43$, $p = 0.01$).

Kidney FcRγ Is Highly Expressed at LN Flare and Confined to the Periglomerular Region

To characterize the immune cell that expresses *FCER1G*, we analyzed the FCER1G encoded protein, Fc receptor gamma chain [FcRγ; (8)] in human LN, and HC kidney tissue by IF microscopic analysis using a specific antibody. The IF analysis revealed that FcRγ is minimally expressed in glomeruli (identified by the podocyte marker, synaptopodin), but abundantly expressed in the PG and TI regions of the LN flare biopsies. The cells expressing FcRγ seemed to have a typical appearance of cellular infiltrate in the PG region, microscopically (**Figure 2**). The IF analysis demonstrated that during LN flare, the PG region is expanded due to the presence of cellular infiltrate, whereas the PG region is thin and without cell infiltration in HC as seen in the merged images of **Figure 2**

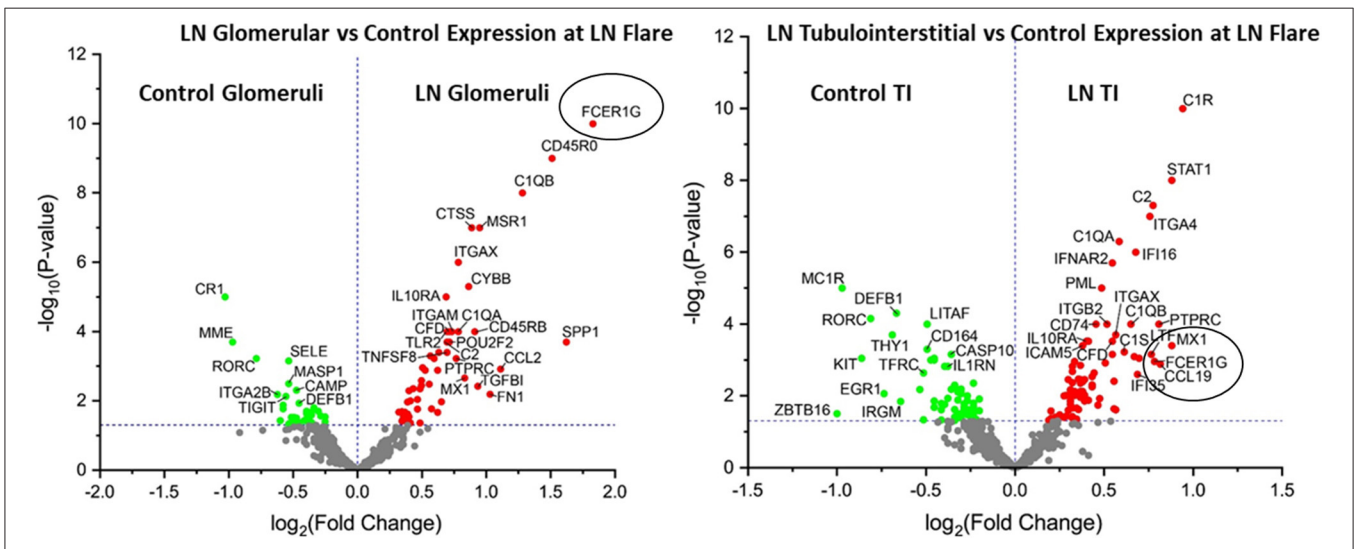


FIGURE 1 | *FCER1G* is one of the top differentially expressed transcript in patients with lupus nephritis (LN). Volcano plot showing expression of *FCER1G* and other top differentially expressed transcripts in human LN glomeruli (G) (left) and tubulointerstitium (right) at the LN flare compared to healthy controls (HCs).

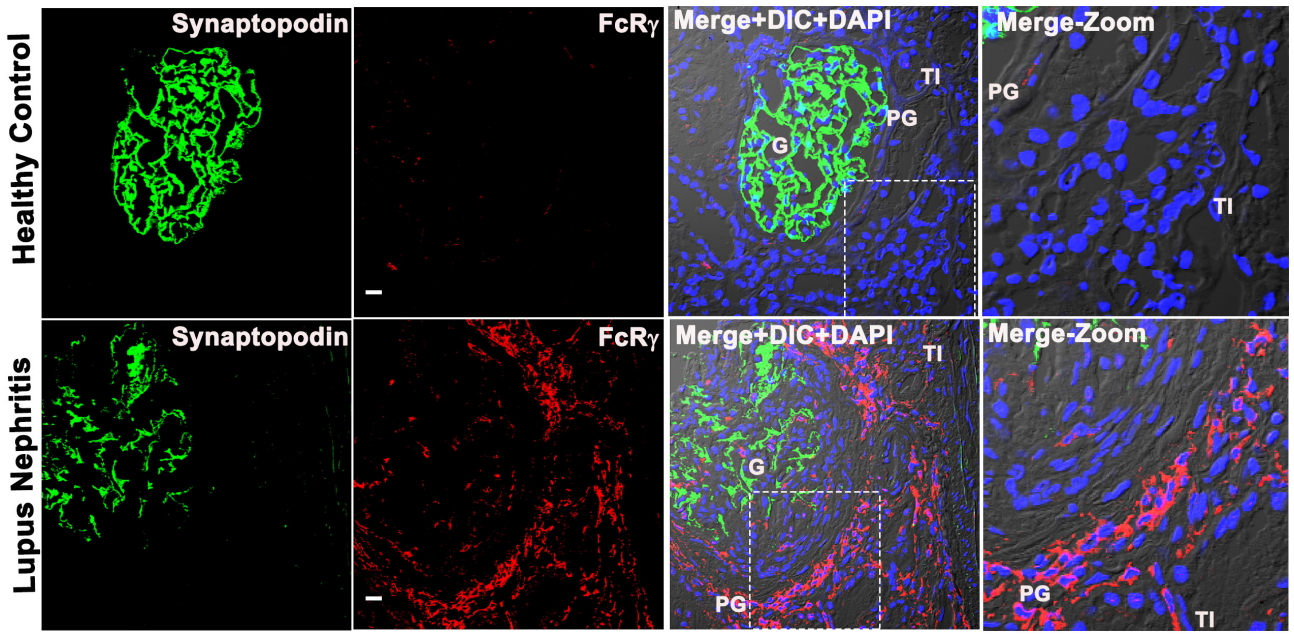


FIGURE 2 | Fc receptor gamma chain is highly expressed in the patient's kidney with LN, and its expression is confined to the periglomerular (PG) region. Three color confocal IF image that is representative of 5 human LN kidney showing the expression of FcRγ (bottom row) in the PG region of the kidney and no expression in the representative of the three HC kidneys (top row). The third column shows merged images of the first 2 columns, i.e., synaptopodin (podocyte marker in green) was used as a landmark for G in the first column, FcRγ (red) in the second column plus differential interference contrast (DIC) microscopy and DAPI staining of nuclei (blue). The fourth column shows the zoomed image of the third column highlighted using a dotted square. The scale bar in the second column represents 20 μm.

and **Supplementary Figure 4**. Most importantly, we saw a weak-to-nil expression of FcRγ in LN and HCs using isotype antibody controls that were processed simultaneously (data not shown). These data support the transcriptomic findings that *FCER1G* codes FcRγ and is abundantly overexpressed in proliferative LN flare.

Macrophage Markers CD11b and CD68 Did Not Colocalize With FcRγ in Human LN Kidney

Based on prior descriptions of interstitial leukocytes in LN (9–11), we predicted the FcRγ expressing cell to be a macrophage. We dual stained biopsy sections with anti-FcRγ antibodies FcRγ antibodies against macrophage markers CD68, CD206, and CD11b. Unexpectedly, staining for M2-specific macrophage markers, CD11b and CD206 [(12); data not shown], showed weak-to-nil expression in the LN kidney. Meanwhile, the pan-macrophage marker CD68 (13) showed glomerular expression; thus, suggesting M1 macrophages were found primarily within the glomeruli. However, CD68 staining did not coincide with PG and TI staining for FcRγ (**Figure 3**). These data demonstrate that FcRγ expressing cells in the kidney are not macrophages. Although antibodies against CD206, CD68, and CD11b gave a weak or no signal in the LN kidney, they did stain Kupffer cells in healthy human liver robustly, confirming the reliability and specificity of the antibodies [(14); data not shown].

FcRγ Colocalized With Conventional Dendritic Cell Marker CD11c and MHCII in LN Kidney

To determine whether the FcRγ expressing cell was a dendritic cell (DC), three-color IF microscopy was done using the conventional DC marker CD11c and MHCII that is known to be highly expressed in all human DCs (15, 16), in addition to the other myeloid cells. The qualitative analysis demonstrated that the FcRγ expression (labeled using anti-FcRγ antibody followed by Alexa-594 dye conjugated secondary antibody (Invitrogen) that showed red emission color in confocal microscopy) colocalized with both CD11c and MHCII. CD11c/MHCII were labeled using CD11c/MHC II antibody followed by Alexa-488 dye conjugated secondary antibody (Invitrogen) showed green emission color in confocal microscopy (**Figure 4**). Colocalization resulted in a yellow color reflecting the presence of both fluorescing FcRγ (red) and CD11c/MHCII (green) to be co-occurring in the same location/cell in the XY dimension (**Figure 4**). The staining pattern of CD11c matched with FcRγ (**Figure 4**) in PG and TI region, and neither antibody stained within the glomeruli. In line with CD11c, MHCII (**Figure 4**, bottom row) also stained strongly in PG, colocalizing with FcRγ. But, in addition to strong PG staining, MHCII also demonstrated weak staining within the glomerulus, suggesting the presence of a weak MHCII expressing glomerular cell that may be a myeloid cell.

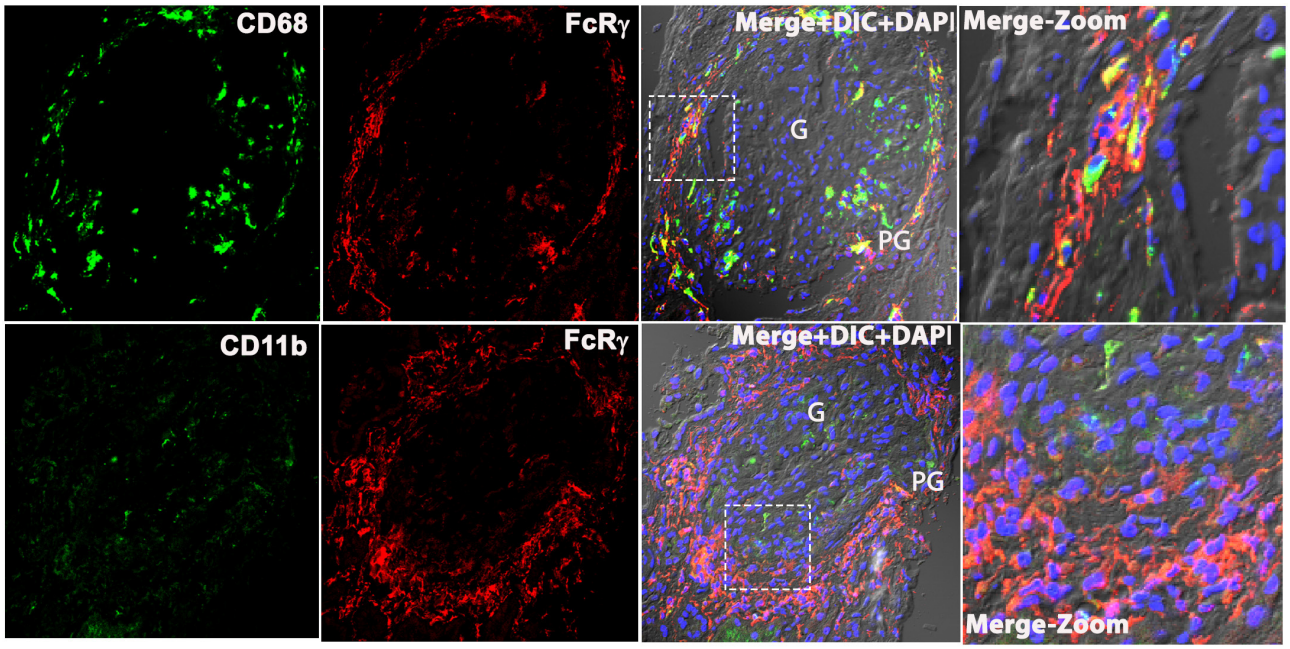


FIGURE 3 | Macrophage markers CD68 and CD11b does not colocalize with FcRγ in the patient's kidney with LN. Three color confocal IF image that is representative of the 3 human LN kidneys showing no colocalization of CD68 (green) with FcRγ (red) (top row) in the PG region in the kidney and CD11b (green) with FcRγ (red) (bottom row). The third column shows merged images of the first 2 columns plus DIC and DAPI staining of nuclei (blue). The fourth column shows the zoomed image of the third column highlighted using a dotted square.

FcRγ Did Not Colocalize With the Plasmacytoid Dendritic Cell Marker CD123, but Colocalized With Monocyte-Derived Dendritic Cell Marker DC-SIGN

To identify which subset of FcRγ-expressing DC is found in the PG region during LN flare, the biopsy tissue was stained for CD123, a specific marker of a plasmacytoid DC (pDC) (16, 17). $CD123^{+}$ cells were found in the TI region (**Figure 5**, top row) but were absent from the glomeruli and the PG region of LN kidneys. $CD123^{+}$ cells were sparse, and CD123 staining in the TI did not align with FcRγ staining. This suggests that the intrarenal FcRγ-expressing DC in LN is not a pDC.

Signal regulatory protein alpha (SIRPα) expression colocalized with FcRγ (**Figure 5**, middle row), suggesting that the PG DCs are not myeloid conventional type 1 DC (cDC1) (16). The remaining possibilities were that the PG DCs are myeloid conventional type 2 DCs (cDCs2) or monocyte-derived DCs (moDCs). To distinguish between these DC subsets, the DC-SIGN expression, a marker specific to moDCs was assessed. DC-SIGN colocalized with FcRγ in the PG region (**Figure 5**, bottom panel) suggesting that the PG DC is a moDC.

FcRγ Colocalized With CD64, CD16, and CD14 in Periglomerular Region of Kidney and Confirmed That DCs Found in the LN Kidney at Flare Are moDCs

To confirm FcRγ expressing DCs are moDCs, they were further characterized by evaluating the presence of additional cell surface markers known to be present on the moDC including CD64, CD16, and CD14. Each of these markers was found in the LN kidney tissue and colocalized with FcRγ (**Supplementary Figure 1**).

FcRγ Colocalized With Previously Identified Circulating infDC Marker CD163 and CD163 Expression Is Overexpressed at LN Flare

Subsequently, colocalization analysis of CD163 with FcRγ was done to determine if the moDC population seen here could be the recently described circulating infDC (18). **Figure 6** confirms CD163 colocalization (green) with FcRγ (red) in the PG region (**Figure 6A**) and the TI (**Figure 6B**) strongly suggesting that the moDC subset present in the kidney during LN flare are indeed infDCs. As infDCs do not express CD5 (18), the kidney tissue was stained for CD5. Although CD5-expressing cells were present in the kidney, they were not located in proximity to the PG region and did not colocalize with FcRγ (**Supplementary Figure 2**), suggesting that these infDC lack CD5 expression.

To quantitate the difference in infDC in LN and HC kidneys, infDC were quantified by measuring the area of CD163 expression after staining with an anti-CD163 monoclonal antibody, and the mean fluorescence intensity of CD163. Using 16 glomerular images from 4 LN flare kidneys, and 18 glomerular images from three HC kidneys, we found a 9- to 21-fold higher level of infDC in LN kidneys compared to HC (**Figure 6C**).

Since infDCs have previously been shown to express CD11b (19, 20), we further analyzed the human LN kidney with rat

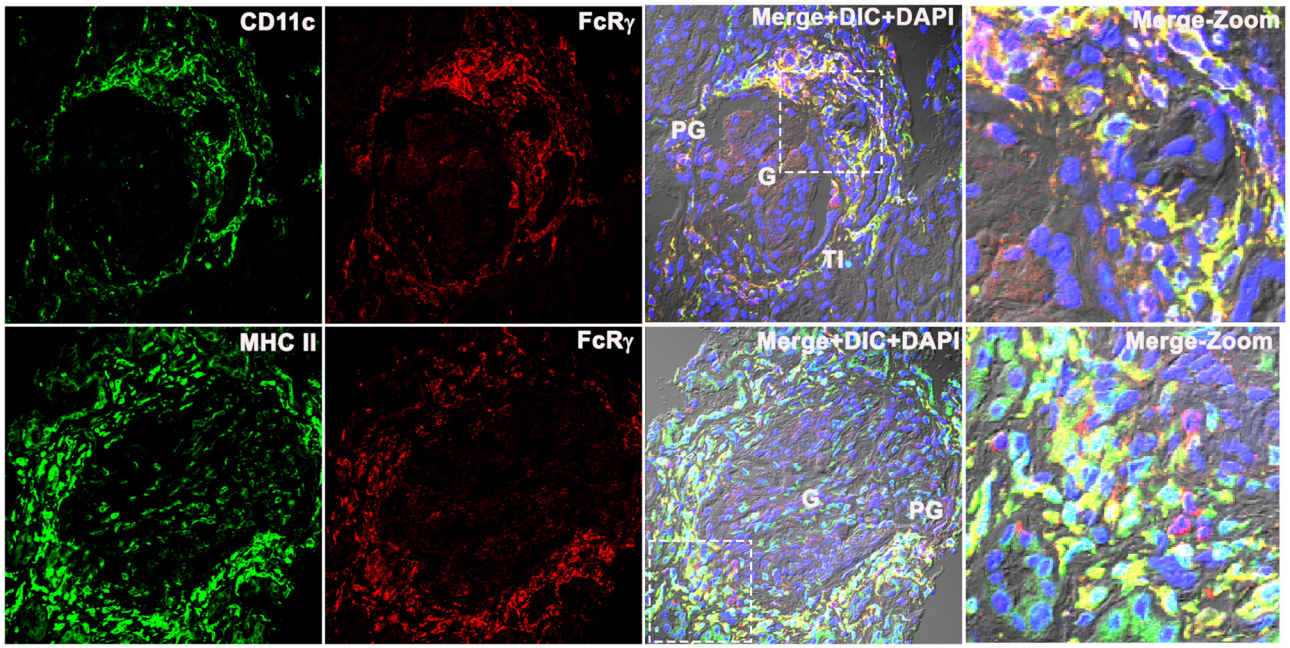


FIGURE 4 | Fc receptor gamma chains colocalize with dendritic cell markers CD11c and MHCII in human LN kidney. Three color IF images that are representative of the 3 human LN kidneys showing colocalization of CD11c (labeled using anti-CD11c antibody followed by Alexa-488 dye conjugated secondary antibody that shows green emission color in confocal microscopy (green) (top row) with FcRγ labeled using anti-FcRγ antibody followed by Alexa-594 conjugated secondary antibody that shows red emission color in confocal microscopy (red). The bottom row shows the colocalization of MHC II labeled using anti-CD11c antibody followed by Alexa-488 dye conjugated secondary antibody that shows green emission color in confocal microscopy (green) with FcRγ labeled using anti- FcRγ antibody followed by Alexa-594 conjugated secondary antibody that shows red emission color in confocal microscopy (red). The third column shows the colocalization of red and green as yellow in the first 2 columns along with DIC and DAPI staining of nuclei (blue). The fourth column shows the zoomed image of the third column highlighted using a dotted square.

anti-human CD11b mAb (clone M1/70) along with anti-CD163 antibody. Consistent with previous findings in this manuscript (**Figure 3**), the clone M1/70 gave a weak-to-nil signal in the infDCs (**Supplementary Figure 3**).

Inflammatory Dendritic Cells Were Present in Close Proximity With T Cells at LN Flare

Lupus nephritis kidney sections were costained with the T cell marker CD3, and antibodies to FcRγ and CD163 to spatially localize T cells and infDCs. CD3 costaining with infDC markers were done in two ways using two different clones of anti-CD3 antibodies (mouse mAb UCHT1 and rabbit mAb SP7) and 2 infDC markers. The CD3 mAb UCHT1 and anti-FcRγ staining showed the presence of $CD3^+$ T cells present adjacent to $FcR\gamma^+$ infDCs in the PG region of the LN kidney (**Figure 7A**). Staining with anti-$CD163^+$ CD3 mAb SP7 (**Figure 7B**) confirmed that infDC were present in close proximity to $CD3^+$ T cells in the human LN kidney. Although the costaining of CD3 with infDC markers showed mainly discrete green and red staining for both cell types, in a few places the red and green staining overlapped.

DISCUSSION

In this study, we identified a novel infDC population infiltrating the kidney at the LN flare. These infDCs infiltrate, localize to the PG and TI spaces, and their expression was 10-fold greater in the LN flare kidney compared to the HCs. The evidence that these are novel is based on their surface markers, being $FcR\gamma^+$, $MHCII^+$, $CD11c^+$, $CD163^+$, $CD5^-$, DC-$SIGN^+$, $CD64^+$, $CD14^+$, $CD16^+$, $SIRP\alpha^+$, $CD206^-$, $CD68^-$, $CD123^-$, $CD3^-$, and $CD11b^-$. Notably, these infDC were present in close approximation to T cells in the PG region of the kidney during LN flare.

The observations from this investigation are supported by previous studies that detected PG DC infiltration in various mouse models of experimental glomerulonephritis (21, 22). However, it is unclear why the infDC settle in the PG space. The previous literature suggests that the renal DC starts in the glomerulus and traverse from mesangium through glomerular tuft and lymphatics to draining lymph nodes in order to present antigen to T cells in the PG (23). In this case, the renal DC may start in the glomerulus but by the time LN becomes clinically evident, these DC have already moved out of the glomerulus and settled in the PG space. Inhibitory signals may then be released and block the cytokine/chemokines required for monocyte differentiation into the infDC, explaining the absence of infDCs from the LN glomeruli at the time of biopsy. Alternatively, it is possible that DC chemotactic factors are released directly from renal tubular cells attracting infiltrating DC (24). We speculate that both occur in response

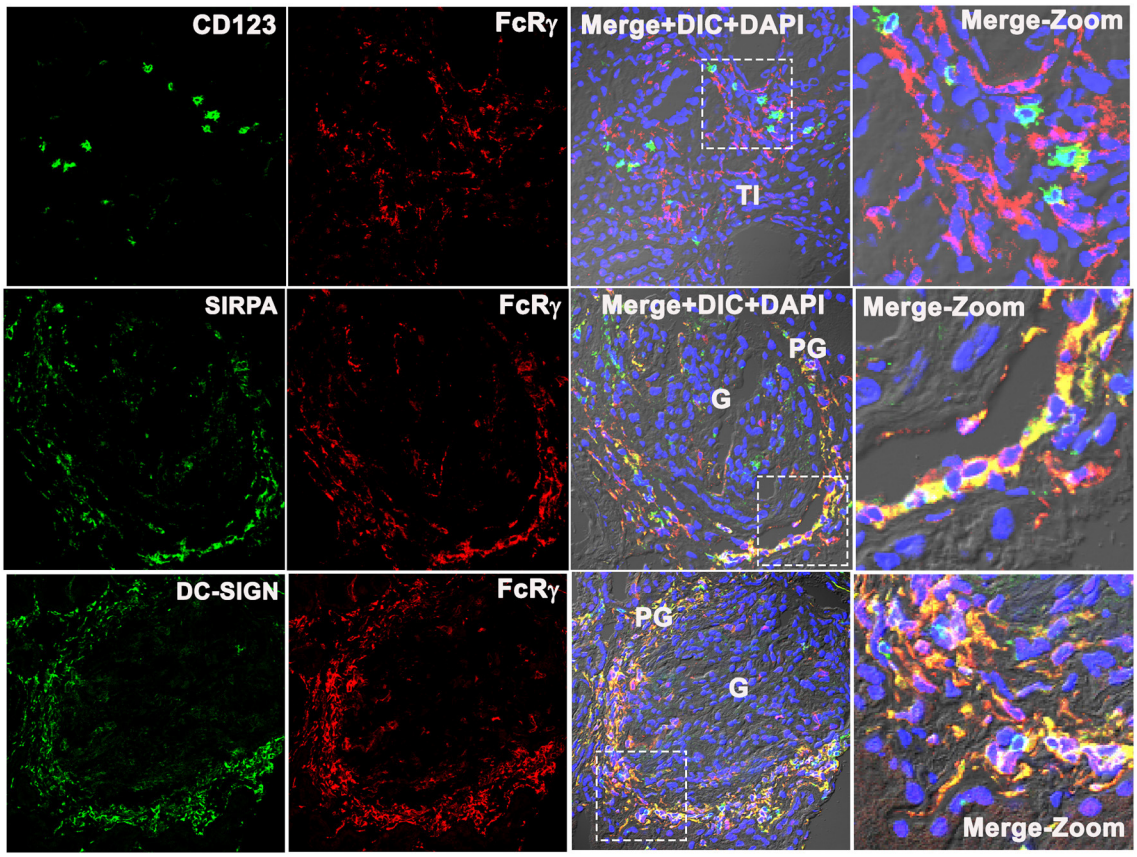


FIGURE 5 | Fc receptor gamma chain does not colocalize with plasmacytoid DC marker CD123, but colocalizes with monocyte-derived DC marker SIRPA and DC-SIGN in the human LN kidney. Three color confocal IF images that are representative of 3 human LN kidneys showing no colocalization of CD123 (green) with Fc receptor gamma chain (FcRγ) (red) (top row) in the TI region of the kidney, but colocalize with monocyte-derived DC cell marker SIRPα (green) (middle row) and DC-SIGN (green) with FcRγ (red) (bottom row) in the PG region. The third column shows merged images of the first 2 columns plus DIC microscopy and DAPI staining of nuclei (blue). The fourth column shows the zoomed image of the third column highlighted using a dotted square.

to inflammatory stimuli resulting in PG and TI accumulation of infDC during LN.

This investigation established that the PG DC are not plasmacytoid or conventional DCs. The expression of monocyte markers CD14, CD64, and CD16 that were reported earlier, confirmed that these PG DCs represent a moDC (16, 25, 26). In the setting of active inflammation or infection, moDCs are termed infDCs (27). Further characterization of these moDCs revealed a unique infDC population not previously described in the human LN. Notably, however, a recent transcriptomic study of peripheral blood immune cells in patients with SLE identified an infDC signature that was $CD163^+$, $CD14^+$, and $CD5^-$, which is similar to the intrarenal infDC population reported here (18). Recently, an extensive evaluation of immune cells was performed using single-cell RNA-Seq from kidney biopsies at the LN flare (28). Several monocyte clusters were identified in this study with one monocyte cluster expressing a $CD163^+$, $CD14^+$, $CD16^+$, $CD64^+$, and DC-$SIGN^+$ signature, similar to the infDC described here. Furthermore, the characterized monocyte lineage was thought to be an infiltrating monocyte subset as it was minimally expressed in healthy kidneys. While these IF studies do not provide a precise measure of the level of lineage marker expression, and this could make identification of transitional stages of the monocyte to DC conversion difficult, these findings do allow for characterization and spatial orientation to inform further quantitative studies.

This novel infDC population also resembles previously reported infDC, from lymph nodes of Listeria-infected mice ($CD64^+CD11c^+MHCII^+$) (27), and gut mucosa of celiac disease (29). InfDCs have also previously been described in human arthritic synovial fluid from patients with rheumatoid arthritis (20). However, the infDC described in the LN kidney differs from synovial fluid infDC; in that, they lack the macrophage markers CD11b and CD206 that are present on synovial fluid infDC. This suggests that the infDC population described here differs from the infDC seen in at least some other autoimmune diseases and may be unique to the kidney during LN flare. Moreover, earlier studies have identified FcεRI [(19, 30); identified using an anti-FcεRIα antibody (eBioscience, Inc., San Diego, CA, USA) for antibody binding site] to be the best marker to distinguish infDC

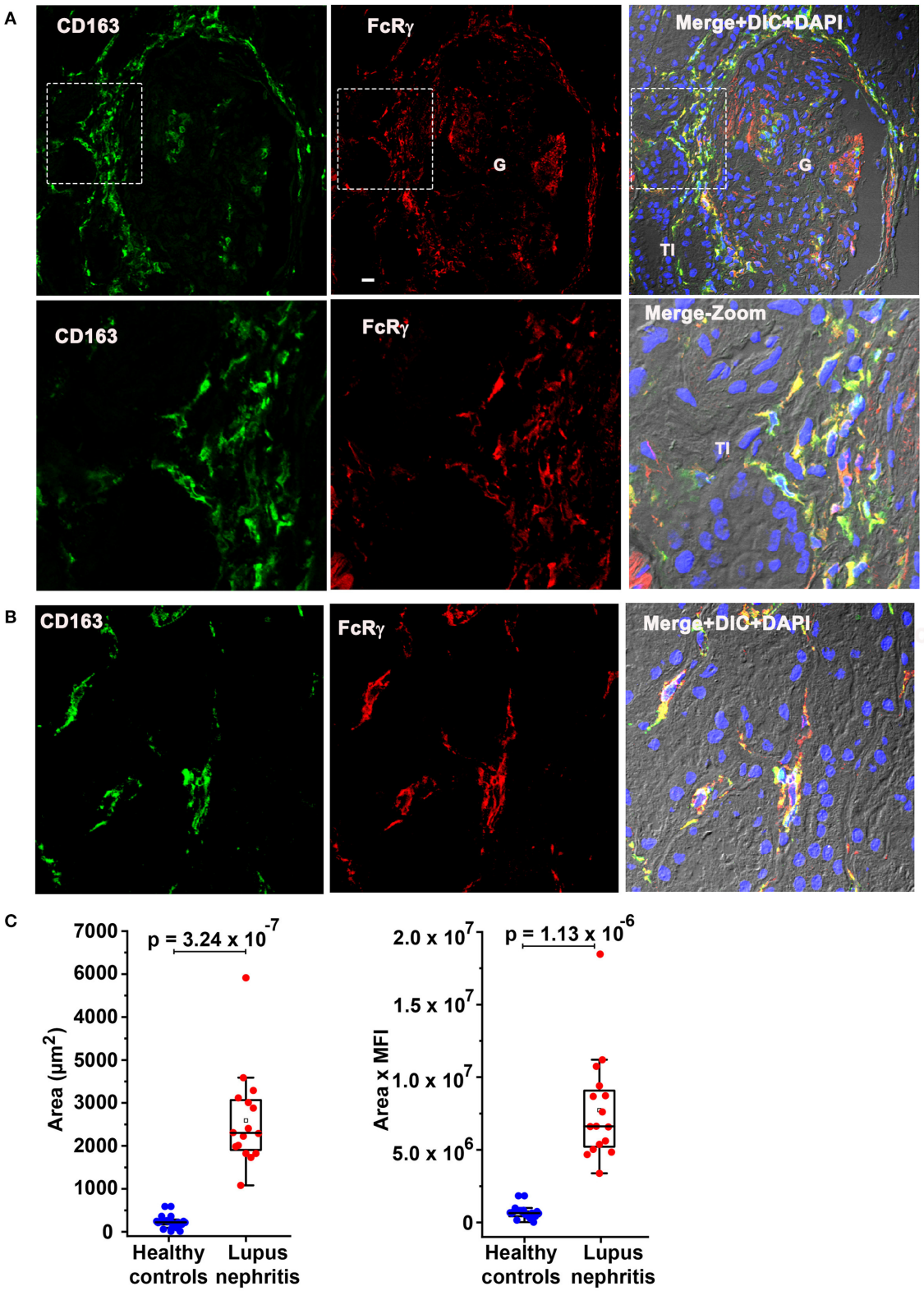


FIGURE 6 | Fc receptor gamma chain colocalizes with previously identified circulating inflammatory dendritic cell (infDC) marker CD163 and CD163 expression is overexpressed in human LN compared to HCs. **(A)** Three color IF images that are representative of 3 human LN kidneys showing colocalization of CD163 in green

(Continued)

FIGURE 6 | (first) with FcRγ in red (middle) in the PG region. The second row shows a zoomed portion of images of the top row highlighted in the dotted square. The third column shows the merging of the first 2 along with DIC and DAPI staining of nuclei (blue). **(B)** Three color IF images that are representative of the 3 human LN kidneys showing colocalization of CD163 in green (first) with FcRγ in red (middle) in the tubulointerstitial (TI) region. **(C)** The bar graph portrays the quantification of infDCs based on CD163 expression using green color from anti-CD163 staining from 18 G images of 3 different HCs and 16 glomeruli images from 4 different LN samples using ImageJ software. The area alone (graph in left) and area × mean fluorescence intensity (graph in left) of CD163 (green) were measured and plotted with mean ± SD. The data were analyzed by unpaired *t*-test (one-tailed) using each measurement, and *p*-values were indicated in the bar graph.

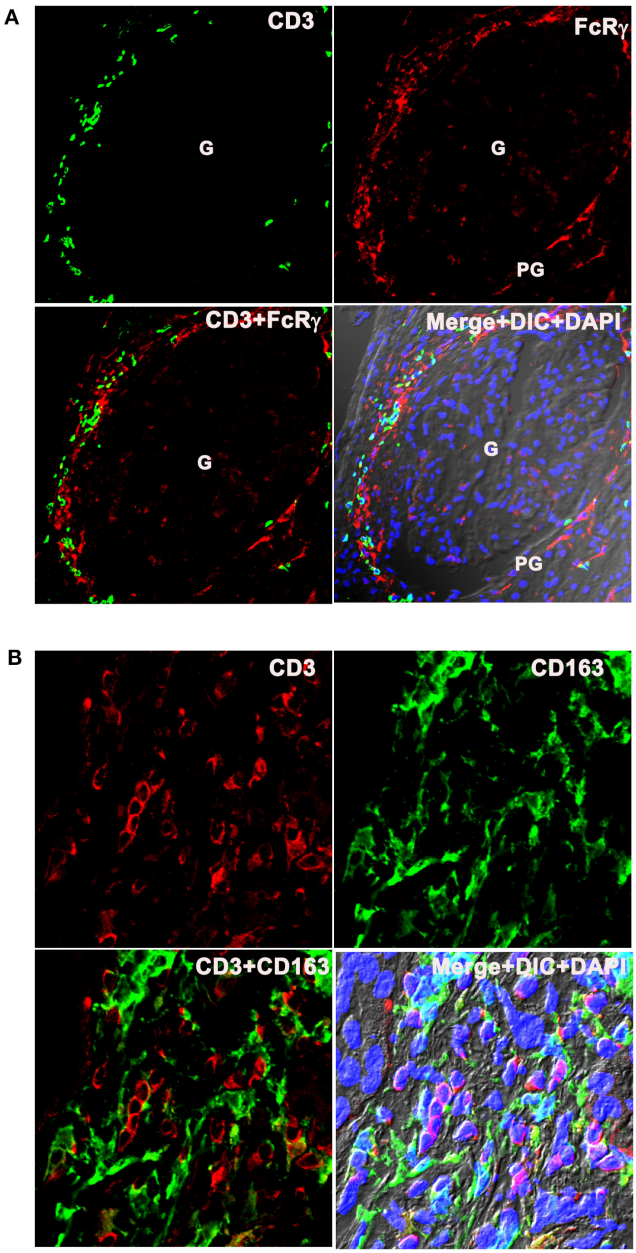


FIGURE 7 | Inflammatory dendritic cells are present in close proximity with T cells at LN flare. **(A)** The top row portrays 3 color IF images that represents three LN kidneys showing the presence of CD3 (Pan T cell marker in green) next to FcRγ (infDC marker in red). The bottom row portrays the merged images of the first 2 columns in the right and merged images of the first 2 columns plus DIC and DAPI staining of nuclei (blue) in the left. **(B)** The top

(Continued)

FIGURE 7 | row portrays 3 color IF images that represent the three different LN kidneys showing the presence of CD3 (pan T cell marker in red) adjacent to CD163 (infDC marker in green) that are highly magnified from the PG region. The bottom row shows the merged images of the first 2 columns in the right and merged images of the first 2 columns plus DIC and DAPI staining of nuclei (blue) in the left.

from macrophages and cDC by flow cytometry. Our studies suggest that FcRγ, a cytoplasmic gamma subunit through which multiple FcRs function, including FcεRI, FcγRI, FcγRIIIa, and FcαRI, as well as other immune receptors like GPVI, OSCAR, and TREM (8), to be a reliable marker for IF studies on patient biopsy samples. It is likely that infDC in the LN kidney express FcεRI, similar to FcγRI and FcγRIIIa (**Supplementary Figure 1**) since the presence of FcRγ indirectly suggests the presence of multiple receptors including FcεRI.

The importance of characterizing the intrarenal cell type expressing CD163 is enhanced by recent findings from our group, revealing that urine CD163 levels were significantly higher in active LN compared to extrarenal SLE or inactive SLE (31). Urine CD163 correlated with disease severity and histologic activity index. While the study by Mejia et al (31). suggested CD163 derives from M2 macrophages, we now suggest that urine CD163 also derives from infDC, supporting the idea that urine CD163 is a biomarker that reflects disease activity in LN.

With regard to the origin of these infDCs, the expression of monocyte markers suggests that infDCs may be differentiated from infiltrated monocytes in the kidney during inflammation caused by various autoimmune stimuli including immune complexes. On the other hand, the presence of $CD163^{+}CD14^{+}$ infDCs in the patients with circulation of lupus (18) suggests that infDC from the peripheral circulation enter the kidney in LN. It is also possible that both mechanisms account for renal infDCs.

The mechanisms by which infDC interact with T cells during human LN are currently unknown. Although the costaining of CD3 along with infDC markers shows mainly discrete populations of both cell types in close proximity (**Figure 7B**), there is also evidence of some overlap suggesting immunological synapse formation and interactions between these infDCs and T cells in the LN kidney. However, T cells are poised to migrate to secondary lymphoid organs; a recent report has shown the presence of immune aggregates organized as tertiary lymphoid structures (TLS) in patients with LN and murine LN kidneys that resemble lymph nodes by gene signatures and cell composition (32). This is consistent with a previous report that identified germinal centers with T- and B-cell aggregates in the LN kidney (33). Considering these findings in the context of identifying

infDCs in the LN kidney suggests that infDCs in the PG and TI may be important drivers of a local adaptive immune response within the LN kidney.

While it is not yet clear whether these T cells are resident T cells or primed T cells, infDCs are known under various disease conditions to have the capacity to trigger the development of major T helper cell subsets, namely Th1, Th2, and Th17 (20, 34, 35). Although, *in vitro* studies suggest infDCs are involved in the initiation and maintenance of Th17 response (19), further studies on the novel LN kidney infDCs are needed because the inflammatory stimulus and the tissue microenvironment determine infDC function *in situ* (36).

CONCLUSION

In conclusion, we identified a novel infDC population that has not been previously described in the LN kidney. These infDCs reside in the PG region and adjacent to infiltrating T cells. Our findings, coupled with recent literature identifying circulating $CD163^+$ infDC in SLE and urine CD163 as a valuable marker of disease activity in LN, suggest infDCs and their T cell partners may be key contributors to driving the local adaptive immune response during active LN. Further study is necessary to define the T cell subsets residing next to the infDCs and understand the mechanisms by which PG infDCs communicate with T cells to drive local inflammation in LN.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP, JS, JT, HS, and LG conducted the experiments. AM, VA, BL, JM-V, and AS contributed to the patient's samples. SP, DB, WJ, BR, and LG contributed to the design of the study and analyzed the data. JZ and LY performed statistical analysis on the nanostring data. SM contributed reagents and performed the statistical analysis. LG, SP, and BR wrote the manuscript. SP and LG conceived the study. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number AR066330.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Brian Kemmenoe at The Ohio State University Campus Microscopy and Imaging Facility for Training with confocal imaging and Paolo Fadda, PharmD from The Genomics Shared Resource (at Comprehensive Cancer Center, Department of Molecular Virology, Immunology, and Medical Genetics at The Ohio State University Wexner Medical Center) who assisted with the nanostring analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.621039/full#supplementary-material

REFERENCES

1. Almaani S, Meara A, Rovin BH. Update on lupus nephritis. *Clin J Am Soc Nephrol*. (2017) 12:825–35. doi: 10.2215/CJN.05780616
2. Rovin BH, Parikh SV. Lupus nephritis: the evolving role of novel therapeutics. *Am J Kidney Dis*. (2014) 63:677–90. doi: 10.1053/j.ajkd.2013.11.023
3. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*. (2008) 26:317–25. doi: 10.1038/nbt1385
4. Golubeva Y, Salcedo R, Mueller C, Liotta LA, Espina V. Laser capture microdissection for protein and NanoString RNA analysis. *Methods Mol Biol*. (2013) 931:213–57. doi: 10.1007/978-1-62703-056-4_12
5. Vaes E, Khan M, Mombaerts P. Statistical analysis of differential gene expression relative to a fold change threshold on nanostring data of mouse odorant receptor genes. *BMC Bioinformatics*. (2014) 15:39. doi: 10.1186/1471-2105-15-39
6. Parikh SV, Malvar A, Song H, Alberton V, Lococo B, Vance J, et al. Characterising the immune profile of the kidney biopsy at lupus nephritis flare differentiates early treatment responders from non-responders. *Lupus Sci Med*. (2015) 2:e000112. doi: 10.1136/lupus-2015-000112
7. Yao Z, Mates JM, Cheplowitz AM, Hammer LP, Maiseyeu A, Phillips GS, et al. Blood-Borne lipopolysaccharide is rapidly eliminated by liver sinusoidal endothelial cells via high-density lipoprotein. *J Immunol*. (2016) 197:2390–9. doi: 10.4049/jimmunol.1600702
8. Brandsma AM, Hogarth PM, Nimmerjahn F, Leusen JH. Clarifying the confusion between cytokine and Fc receptor "common gamma chain". *Immunity*. (2016) 45:225–6. doi: 10.1016/j.immuni.2016.07.006
9. Couzi L, Merville P, Deminiere C, Moreau JF, Combe C, Pellegrin JL, et al. Predominance of CD8+ T lymphocytes among periglomerular infiltrating cells and link to the prognosis of class III and class IV lupus nephritis. *Arthritis Rheum*. (2007) 56:2362–70. doi: 10.1002/art.22654
10. Winchester R, Wiesendanger M, Zhang HZ, Steshenko V, Peterson K, Geraldino-Pardilla L, et al. Immunologic characteristics of intrarenal T cells: trafficking of expanded CD8+ T cell beta-chain clonotypes in progressive lupus nephritis. *Arthritis Rheum*. (2012) 64:1589–600. doi: 10.1002/art.33488
11. Tilstra JS, Avery L, Menk AV, Gordon RA, Smita S, Kane LP, et al. Kidney-infiltrating T cells in murine lupus nephritis are metabolically and functionally exhausted. *J Clin Invest*. (2018) 128:4884–97. doi: 10.1172/JCI120859
12. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E. Phenotypic, functional, and plasticity features of classical and alternatively activated

human macrophages. *Am J Respir Cell Mol Biol*. (2015) 53:676–88. doi: 10.1165/rcmb.2015-0012OC
13. Bertani FR, Mozetic P, Fioramonti M, Iuliani M, Ribelli G, Pantano F, et al. Classification of M1/M2-polarized human macrophages by label-free hyperspectral reflectance confocal microscopy and multivariate analysis. *Sci Rep*. (2017) 7:8965. doi: 10.1038/s41598-017-08121-8
14. Ganesan LP, Kim J, Wu Y, Mohanty S, Phillips GS, Birmingham DJ, et al. FcgammaRIIb on liver sinusoidal endothelium clears small immune complexes. *J Immunol*. (2012) 189:4981–8. doi: 10.4049/jimmunol.1202017
15. Collin M, Mcgovern N, Haniffa M. Human dendritic cell subsets. *Immunology*. (2013) 140:22–30. doi: 10.1111/imm.12117
16. Collin M, Bigley V. Human dendritic cell subsets: an update. *Immunology*. (2018) 154:3–20. doi: 10.1111/imm.12888
17. Jegalian AG, Facchetti F, Jaffe ES. Plasmacytoid dendritic cells: physiologic roles and pathologic states. *Adv Anat Pathol*. (2009) 16:392–404. doi: 10.1097/PAP.0b013e3181bb6bc2
18. Dutertre CA, Becht E, Irac SE, Khalilnezhad A, Narang V, Khalilnezhad S, et al. Single-Cell analysis of human mononuclear phagocytes reveals subset-defining markers and identifies circulating inflammatory dendritic cells. *Immunity*. (2019) 51:573–89.e8. doi: 10.1016/j.immuni.2019.08.008
19. Segura E, Amigorena S. Identification of human inflammatory dendritic cells. *Oncoimmunology*. (2013) 2:e23851. doi: 10.4161/onci.23851
20. Segura E, Touzot M, Bohineust A, Cappuccio A, Chiocchia G, Hosmalin A, et al. Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity*. (2013) 38:336–48. doi: 10.1016/j.immuni.2012.10.018
21. Janssen U, Ostendorf T, Gaertner S, Eitner F, Hedrich HJ, Assmann KJ, et al. Improved survival and amelioration of nephrotoxic nephritis in intercellular adhesion molecule-1 knockout mice. *J Am Soc Nephrol*. (1998) 9:1805–14.
22. Kruger T, Benke D, Eitner F, Lang A, Wirtz M, Hamilton-Williams EE, et al. Identification and functional characterization of dendritic cells in the healthy murine kidney and in experimental glomerulonephritis. *J Am Soc Nephrol*. (2004) 15:613–21. doi: 10.1097/01.ASN.0000114553.36258.91
23. Heymann F, Meyer-Schwesinger C, Hamilton-Williams EE, Hammerich L, Panzer U, Kaden S, et al. Kidney dendritic cell activation is required for progression of renal disease in a mouse model of glomerular injury. *J Clin Invest*. (2009) 119:1286–97. doi: 10.1172/JCI38399
24. Fiore N, Castellano G, Blasi A, Capobianco C, Loverre A, Montinaro V, et al. Immature myeloid and plasmacytoid dendritic cells infiltrate renal tubulointerstitium in patients with lupus nephritis. *Mol Immunol*. (2008) 45:259–65. doi: 10.1016/j.molimm.2007.04.029
25. Langlet C, Tamoutounour S, Henri S, Luche H, Ardouin L, Grégoire C, et al. CD64 expression distinguishes monocyte-derived and conventional dendritic cells and reveals their distinct role during intramuscular immunization. *J Immunol*. (2012) 188:1751–60. doi: 10.4049/jimmunol.1102744
26. Tamoutounour S, Guilliams M, Montanana Sanchis F, Liu H, Terhorst D, Malosse C, et al. Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity*. (2013) 39:925–38. doi: 10.1016/j.immuni.2013.10.004
27. Min J, Yang D, Kim M, Haam K, Yoo A, Choi JH, et al. Inflammation induces two types of inflammatory dendritic cells in inflamed lymph nodes. *Exp Mol Med*. (2018) 50:e458. doi: 10.1038/emm.2017.292
28. Arazi A, Rao DA, Berthier CC, Davidson A, Liu Y, Hoover PJ, et al. The immune cell landscape in kidneys of patients with lupus nephritis. *Nat Immunol*. (2019) 20:902–14. doi: 10.1038/s41590-019-0398-x
29. Beitnes AC, Ráki M, Brottveit M, Lundin KE, Jahnsen FL, Sollid LM. Rapid accumulation of CD14+CD11c+ dendritic cells in gut mucosa of celiac disease after *in vivo* gluten challenge. *PLoS ONE*. (2012) 7:e33556. doi: 10.1371/journal.pone.0033556
30. Segura E, Amigorena S. Inflammatory dendritic cells in mice and humans. *Trends Immunol*. (2013) 34:440–5. doi: 10.1016/j.it.2013.06.001
31. Mejia-Vilet JM, Zhang XL, Cruz C, Cano-Verduzco ML, Shapiro JP, Nagaraja HN, et al. Urinary soluble CD163: a novel noninvasive biomarker of activity for lupus nephritis. *J Am Soc Nephrol*. (2020) 31:1335–47. doi: 10.1681/ASN.2019121285
32. Dorraji SE, Kanapathippillai P, Hovd AK, Stenersrød MR, Horvei KD, Ursvik A, et al. Kidney tertiary lymphoid structures in lupus nephritis develop into large interconnected networks and resemble lymph nodes in gene signature. *Am J Pathol*. (2020) 190:2203–25. doi: 10.1016/j.ajpath.2020.07.015
33. Chang A, Henderson SG, Brandt D, Liu N, Guttikonda R, Hsieh C, et al. *In situ* B cell-mediated immune responses and tubulointerstitial inflammation in human lupus nephritis. *J Immunol*. (2011) 186:1849–60. doi: 10.4049/jimmunol.1001983
34. Nakano H, Lin KL, Yanagita M, Charbonneau C, Cook DN, Kakiuchi T, et al. Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nat Immunol*. (2009) 10:394–402. doi: 10.1038/ni.1707
35. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, et al. Inflammatory dendritic cells–not basophils–are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med*. (2010) 207:2097–111. doi: 10.1084/jem.20101563
36. Hespel C, Moser M. Role of inflammatory dendritic cells in innate and adaptive immunity. *Eur J Immunol*. (2012) 42:2535–43. doi: 10.1002/eji.201242480

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

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 19 February 2021
doi: 10.3389/fimmu.2021.624685

Antigen-Specific Treatment Modalities in MS: The Past, the Present, and the Future

Judith Derdelinckx[1,2*], *Patrick Cras*[2,3], *Zwi N. Berneman*[1,4] *and Nathalie Cools*[1,4]

[1] Laboratory of Experimental Hematology, Vaccine and Infectious Disease Institute (VaxInfectio), Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium, [2] Division of Neurology, Antwerp University Hospital, Edegem, Belgium, [3] Born Bunge Institute, Translational Neurosciences, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium, [4] Center for Cell Therapy and Regenerative Medicine, Antwerp University Hospital, Edegem, Belgium

Antigen-specific therapy for multiple sclerosis may lead to a more effective therapy by induction of tolerance to a wide range of myelin-derived antigens without hampering the normal surveillance and effector function of the immune system. Numerous attempts to restore tolerance toward myelin-derived antigens have been made over the past decades, both in animal models of multiple sclerosis and in clinical trials for multiple sclerosis patients. In this review, we will give an overview of the current approaches for antigen-specific therapy that are in clinical development for multiple sclerosis as well provide an insight into the challenges for future antigen-specific treatment strategies for multiple sclerosis.

Keywords: multiple sclerosis, antigen-specific therapy, tolerance induction, myelin, experimental autoimmune encephalomyelitis

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Daniel Benitez-Ribas,
Hospital Clínic de Barcelona, Spain
Jun Peng,
Shandong University, China
Pablo Villoslada,
Institut de Recerca Biomèdica August Pi i Sunyer (IDIBAPS), Spain

***Correspondence:**
Judith Derdelinckx
Judith.derdelinckx@uantwerpen.be

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *31 October 2020*
Accepted: *04 January 2021*
Published: *19 February 2021*

Citation:
Derdelinckx J, Cras P, Berneman ZN and Cools N (2021) Antigen-Specific Treatment Modalities in MS: The Past, the Present, and the Future. Front. Immunol. 12:624685. doi: 10.3389/fimmu.2021.624685

INTRODUCTION

In autoimmune diseases, the immune system is derailed generating immunity against self. In the particular case of multiple sclerosis (MS), there are strong indications that the loss of tolerance is directed toward various myelin proteins, including myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and proteolipid protein (PLP) (1). Although the exact cause for this breach in tolerance is not yet known, it has been suggested that myelin-reactive CD4+ T lymphocytes, both of the T helper 1 (Th1) and T helper 17 (Th17) type, play a central role in the pathogenesis of MS (1–4). For instance, this is evidenced by the encephalitogenic capacity of CD4+ myelin-reactive T cells following passive transfer in experimental autoimmune encephalomyelitis (EAE) animal models (5, 6). Additionally, the fact that the strongest genetic risk factor for MS lies within the major histocompatibility complex (MHC) class II gene further underscores the importance of CD4+ T cells in MS pathogenesis (1, 7). More recently, the involvement of additional effector cells in the myelin-directed autoimmune reaction has been proposed, including myelin-reactive CD8+ T cells and B cells (4) (**Box 1**). Altogether, a complex autoimmune cascade, rather than a single culprit autoimmune response, appears to be driving MS pathogenesis, complicating the development of a targeted antigen-specific therapy for MS.

The strong increase in knowledge regarding the pathogenesis of MS has resulted in a significant expansion of the treatment armamentarium for MS over the last years. This resulted in a wide range of disease-modifying therapeutics with varying efficacy in reducing inflammation and relapse rate.

However, these therapies are accompanied by various side effects, including opportunistic infections, because of the non-disease antigen-specific mode of action resulting in a more general immune modulation or immune suppression. Hence, an ideal therapy approach for MS would aim to restore the dysregulated myelin-directed immune response without hampering the normal surveillance and effector function of the immune system (**Box 1**).

In this review, we will first give an overview of the current approaches for antigen-specific therapy that are in clinical development for MS, summarizing the results of several phase I, II and III clinical trials. In the second part of this review, we will provide an insight into the challenges for future antigen-specific treatment strategies for MS and summarize the possible solutions for these challenges that are currently being evaluated in a preclinical setting.

BOX 1 | The immune pathogenesis of multiple sclerosis.

MS is considered to be a predominantly T cell-mediated autoimmune disease (118), directed toward various myelin-derived antigens, including myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and αB-crystallin (1), that are expressed in the CNS. This autoimmunity is mostly mediated by CD4+ T cells, in particular T helper 1 (Th1) and Th17 cells (3), and involves further effectuation of an immune cascade involving CD8+ T cells, B cells, and NK cells. The exact mechanism by which these autoreactive T cells are initiated, has not been fully elucidated. As reviewed by Hemmer et al., two main hypotheses have been suggested for the immune-mediated development of demyelinating lesions (2). The first hypothesis—the so-called outside-in hypothesis—is based on peripheral activation of autoreactive CD4+ T cells recognizing CNS-derived antigens, *e.g.*, due to infection-related molecular mimicry or bystander activation (119–122). Alternatively, the inside-out hypothesis states that the initial pathogenic event takes place within the CNS, namely primary oligodendrocyte damage leading to leakage of CNS antigens to the periphery and activation of autoreactive T lymphocytes in the peripheral lymph nodes (123). However, the inside-out hypothesis is controversial, with both evidence in favor (124) and against (125) primary oligodendrocyte damage as the initiating trigger for CNS auto-immunity. Hence, the origin of the autoimmune response in MS remains a matter of debate. Nonetheless, whether the initial pathogenic event takes place in the CNS or in the periphery, one of the key elements in the immune pathogenesis of MS is the escape of autoreactive T cells from tolerance control mechanisms. This allows activated encephalitogenic CD4+ T cells to migrate across the blood-brain barrier (BBB), followed by their reactivation with autoantigens in the perivascular space (126) and their release of inflammatory mediators which activate microglia (2) (**Figure 1**). These cells will, in turn, effectuate tissue damage and produce various chemokines leading to further recruitment of effector and antigen-presenting cells (APC).

Used abbreviations: APC, antigen-presenting cell; IFN, interferon; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; APL, altered peptide ligand; Treg, regulatory T cell.

ANTIGEN-SPECIFIC TREATMENT IN MS: RESULTS FROM CLINICAL TRIALS

Peptides and Altered Peptide Ligands

Peptide-based therapy aims to restore tolerance to specific peptides or peptide mixes by repeated administration through various routes. In parallel to hyposensitization therapy for allergy, this repeated exposure to auto-antigen induces immunological alterations, including a cytokine shift away from the autoimmune Th1/Th17 profile and induction of IL-10-secreting regulatory T cells (Treg) (8–10). Disease-related

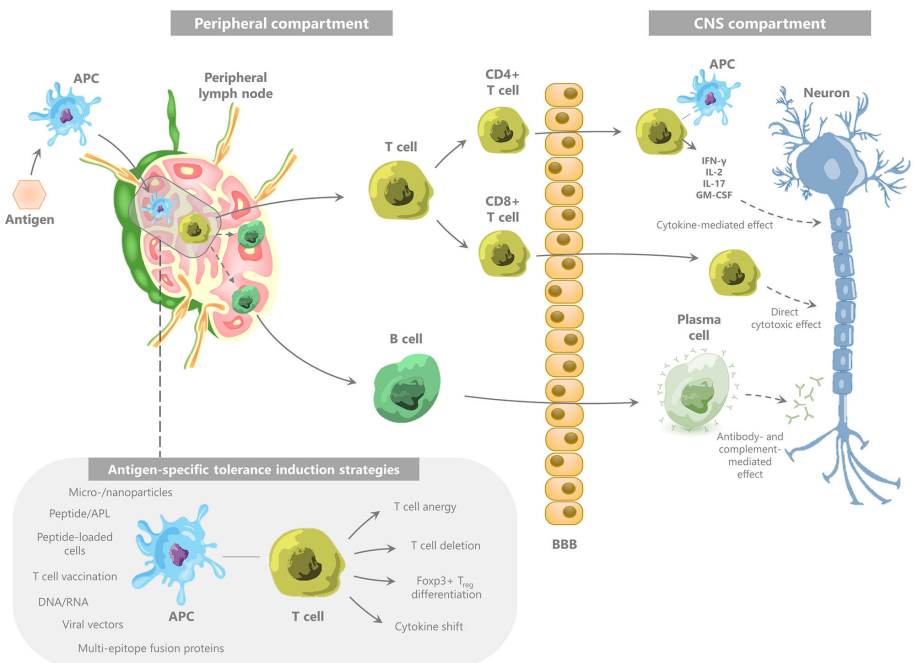


FIGURE 1 | The immune pathogenesis of multiple sclerosis and the concept of myelin-specific tolerance induction.

peptides can be selected by different means, including i) elution from peptide-MHC complexes (representing naturally processed peptides), ii) selection of immune dominant peptide responses by use of reactivity screening assays, or iii) prediction with computer algorithms or databases (11, 12). In addition to the use of classical peptides, altered peptide ligands (APL) can be generated by subtle modification of peptide structure, mostly by amino acid substitutions at the T cell receptor (TCR) binding site. These modifications impair T cell function following TCR-ligand interaction, which can further modulate antigen-specific T cell responses. The therapeutic potential of APL has historically been underlined by the effectiveness of glatiramer acetate, which – among other working mechanisms—acts as an APL for MBP_{82-100}, causing a shift in the MBP response from a Th1 to Th2 cytokine profile [as reviewed by Schrempf et al. (13)].

Peptide- and APL-based therapy is a straightforward yet versatile approach and therefore has been the focus of many clinical trials in MS. An overview of the pivotal clinical trials focusing on peptide therapy in MS can be found in **Table 1**, which we will concisely discuss.

Peptide Therapy

Tolerance induction using peptide therapy was one of the first attempts for antigen-specific treatment for MS, with the first results on efficacy being available from phase II clinical trials at the end of the 90's. In a phase II clinical trial, 30 relapsing remitting MS (RR-MS) patients were treated orally with bovine myelin or with control protein (14, 22). Although only 40% of patients in the group treated with myelin protein had at least one major exacerbation as compared to 80% of patients in the control group (p=0.06) (14), no conclusions regarding efficacy could be made based on these small numbers of patients (22).

Next, a placebo-controlled phase II clinical trial with intravenous administration of high doses of MBP_{82-92} was initiated by Warren et al. in 2006 (16). In this trial, 32 primary or secondary progressive MS (SP-MS) patients were treated with MBP_{82-92} intravenously every 6 months. No difference was found between treatment or placebo group in the primary endpoint, expanded disability status scale (EDSS) progression at 24 months. However, a subgroup analysis of the human leukocyte antigen (HLA)-DR2+ or DR4+ participants (20 subjects) revealed a significantly lower proportion of patients with sustained progression at 24 months in the treatment group (0/10) compared to the placebo group (6/10, p=0.01). Based on the finding that patients responded better depending on their HLA haplotype, a larger phase III clinical trial was initiated in DR2+ or DR4+ SP-MS patients (17). However, this phase III placebo-controlled trial in 612 study subjects failed to meet its primary outcome, *i.e.*, time to progression by ≥1.0 EDSS point, or ≥0.5 point if baseline EDSS was 5.5 or higher (17).

Within the context of the finding of an association between HLA haplotype and clinical effect of peptide vaccination, two phase I clinical trials have been performed using fusion products with HLA molecules. First, in 2000, a phase I dose-escalating clinical trial with intravenously administered MBP_{84-102} complexed to HLA-DR2 (AG284) in 33 HLA-DR2+ secondary MS patients was initiated, showing a favorable safety profile but no effect on clinical and radiological secondary outcome measures (15). Secondly, in 2012, a phase I dose-escalation clinical trial in 34 HLA-DR2+ MS-patients demonstrated that a fusion product of the two outer domains of HLA-DR2 with MOG_{35-55} was well tolerated up to a dose of 60 mg intravenously without increase in MS disease activity (18).

In 2013, Walczak et al. reported the results of their clinical trial with transdermal myelin peptide treatment (19). In their placebo-controlled study, 30 patients with active RR-MS were treated with a skin patch, either containing a mixture of three myelin-derived peptides (MBP_{85-99}, MOG_{35-55}, and $PLP_{139-155}$) or phosphate-buffered saline (PBS). A 66.5% reduction in the cumulative number of gadolinium (Gd)-enhancing lesions compared with placebo treatment (p=0.02) was found on 3-monthly magnetic resonance imaging (MRI) scans during the first year of treatment.

In 2015, Streeter et al. reported results from a phase I clinical trial in SP-MS patients (20), which were treated with a mix of 4 MBP-derived "apitopes" or antigen processing-independent epitopes (MBP_{30-44}, $MBP_{131-145}$, $MBP_{140-154}$, and MBP_{83-99}) called ATX-MS-1467. These apitopes mimic the naturally processed T cell epitope, binding directly onto MHC class-II on immature dendritic cells (DC). This was considered to be of importance since it was previously demonstrated that attempts to induce tolerance toward a non-naturally processed epitope, *i.e.*, cryptic epitope, were not able to prevent EAE (23). Six SP-MS patients were treated with weekly to biweekly intradermal administrations of ATX-MS-1467, each receiving a dose escalation from 25 to 800 μg (20). Treatment was well-tolerated, with no major side effects. The phase Ib study, aiming to determine the optimal route of administration, showed a 73% decrease in new or persisting Gd-enhancing T1 lesions from baseline to week 16 (end of the treatment period) in the intradermal group, returning to baseline levels at week 48 (end of the off-treatment period), whereas no MRI differences could be detected in the subcutaneously treated group (24).

Immunomonitoring was performed in several of these clinical trials, demonstrating reduction in the frequency (14) and the proliferative capacity (25) of myelin-reactive T cells, a peripheral blood cytokine shift toward anti-inflammatory interleukin (IL)-10 secretion (25) and induction of myelin-specific transforming factor β (TGF-β)-secreting regulatory T cells (Treg) (26–28) following myelin peptide treatment.

In conclusion, clinical trials with peptide-based treatment have yielded both promising and disappointing results. Differences in administration route, patient population, and single-peptide- *versus* multi-peptide-based treatment may play a role in these contrasting results. At the moment, research into peptide-based therapy is continuing in MS. Currently under investigation is Neurovax®, a vaccine consisting of peptides derived from the T cell receptor (TCR) of pathogenic T cell clones of MS patients (29–31). Intramuscular administration of this vaccine aims to specifically modulate autoreactive T cells recognizing these peptides. Phase I clinical trials with this peptide product are currently ongoing in SP-MS and pediatric MS (NCT02200718, NCT02149706, and NCT02057159).

TABLE 1 | Overview of the clinical trials using peptide therapy.

Author and year	Peptide	Trial design	Route of administration and timing	Patient population	Primary end point	Results
Weiner et al. 1993 (14)	Bovine myelin	Placebo-controlled phase II	Oral, daily	30 RR-MS patients	Number of severe exacerbations	Fewer severe exacerbations in treated group (6/15 versus 12/15, p=0.06)
Goodkin et al. 2000 (15)	MBP_{84-102} complexed to HLA-DR2	Placebo-controlled phase I	Intravenous, on day 0, 2, and 4	33 HLA-DR2+ SP-MS	Safety profile	Favorable safety profile but no effect on clinical and radiological secondary outcome measures
Warren et al. 2006 (16)	MBP_{82-92}	Placebo-controlled phase II	Intravenous, every 6 months	32 PP-MS or SP-MS patients	EDSS progression at 24 months	No significant difference in total population In HLA-DR2- of HLA-DR4-positive subgroup: significant lower proportion of patients with sustained progression (0/10 *versus* 6/10, p=0.01)
Freedman et al. 2011 (17)	MBP_{82-92}	Placebo-controlled phase III	Intravenous, every 6 months	528 DR2- or DR4-positive SP-MS patients 110 DR2- and DR4-negative SP-MS patients	Time to confirmed EDSS progression	No significant differences
Yadav et al. 2012 (18)	MOG_{35-55} complexed to HLA-DR2	Phase I	Intravenous, single injection	34 HLA-DR2+ MS-patients	Safety profile	Well tolerated up to a dose of 60 mg without increase in MS disease activity
Walczak et al. 2013 (19)	MBP_{85-99}, MOG_{35-55} and $PLP_{139-155}$	Placebo-controlled phase I/II	Transdermal, continuous	30 RR-MS patients	Cumulative number of active Gd+ lesions per patient per scan during the year of the study	66.5% reduction in the cumulative number of Gd-enhancing lesions compared with placebo treatment (p=0.02)
Streeter et al. 2015 (20)	ATX-MS-1467 (MBP_{30-44}, $MBP_{131-145}$, $MBP_{140-154}$ and MBP_{83-99})	Phase I	Intradermal, weekly to biweekly	6 SP-MS patients	Safety profile	Safe and well-tolerated
Chataway et al. 2018 (21)	ATX-MS-1467	Phase Ib	Intradermal *versus* subcutaneous, weekly to biweekly	43 DRB1*15-positive RR-MS patients	Safety profile	Safe and well-tolerated. 73% decrease in new or persisting Gd-enhancing T1 lesions from baseline to week 16 (end of the treatment period) in the intradermal group versus no MRI differences in the subcutaneous group
	ATX-MS-1467	Phase IIa	Intradermal, weekly to biweekly, with a shorter titration period and longer high-dose treatment period	37 DRB1*15-positive RR-MS patients	Number of Gd+ lesions	Significant decrease in number and volume of new or persisting gadolinium-enhancing lesions, both on-treatment and post-treatment

RR-MS, relapsing-remitting multiple sclerosis; HLA, human leukocyte antigen; SP-MS, secondary-progressive multiple sclerosis; EDSS, Expanded Disability Status Scale; Gd, gadolinium; MRI, magnetic resonance imaging.

Altered Peptide Ligands

Several authors demonstrated the prevention of EAE development in rodents by administration of APL for MBP (32–37) or PLP (38–40) peptides. However, clinical translation appeared to be less unequivocal. A phase II clinical trial assessing the safety and efficacy of weekly subcutaneous administration of an APL of $MBP_{83–99}$ (CGP77116) was halted prematurely after treatment of 8 patients because of treatment-related occurrence of MS exacerbations in 3 patients (41). Treatment with CGP77116 carried the risk of expansion of encephalitogenic $MBP_{83–99}$-reactive T cells, as demonstrated by a strong increase in frequency of $MBP_{83–99}$- and CGP77116-reactive T cells in peripheral blood and cerebrospinal fluid (CSF) in two of the three patients during disease exacerbation. In the same year, a second clinical trial with a different APL of $MBP_{83–99}$ (NBI5788) was suspended after hypersensitivity reactions were observed in 9.1% of treated patients (42), even though NBI5788 was shown to be safe in a phase I study (43). Hypersensitivity was Th2-driven and arose in most patients after more than 10 administrations. Nonetheless, the volume and number of Gd-enhancing lesions 4 months after the first administration was reduced in the group of patients treated with the lowest dose of 5 mg of NBI5788 (42). Hence, induction of Th2 responses toward myelin antigens appeared to be a double-edged sword, with both beneficial and adverse effects. Similar immediate hypersensitivity reactions have been reported for glatiramer acetate, making the authors conclude that APL might be a new class of therapeutics for MS, but with the need to regulate the strength of the Th2 response (42). Nevertheless, despite the success of glatiramer acetate, no clinical trials using APL have been initiated since then, even though preclinical work on APL in EAE models still continues (44–46).

Peptide-Loaded Cell Therapies

A phase I dose escalation clinical study was performed by Lutterotti et al., using autologous peripheral blood mononuclear cells (PBMC) coupled to 7 myelin peptides ($MOG_{1–20}$, $MOG_{35–55}$, $MBP_{13–32}$, $MBP_{83–99}$, $MBP_{111–129}$, $MBP_{146–170}$, and $PLP_{139–154}$) in the presence of the chemical cross-linker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (47). Seven RR-MS and 2 SP-MS patients were treated with doses ranged from $1x10^3$ to $3x10^9$ antigen-coupled PBMC, administered in one single intravenous infusion (47). No major side effects were reported. Moreover, myelin-specific T cell responses were reduced 3 months after treatment in the four patients receiving highest doses ($\geq 1x10^9$ myelin-coupled PBMC). Two mechanisms appear to be driving tolerance induction through EDC-fixed peptide-loaded carrier cells, which are themselves deprived of their cellular function following fixation. Based on EAE data, a first mechanism consists of induction of apoptosis in myelin-reactive T cells upon antigen presentation without costimulation by the EDC-fixed carrier cells (48). In addition, a contribution of secondary cross-tolerance induction by presentation of peptides by host antigen-presenting cells (APC) following uptake and processing of the peptide-loaded carrier cells was demonstrated (48). Given the promising results of this phase I clinical trial, a phase I/II clinical trial focusing on peptide-loaded red blood cells, called ETIMSred, was initiated recently (49).

In addition to the use of fixed carrier cells, peptide-loaded cell therapy strategies can make use of viable APC as carrier cells to add a direct tolerogenic property to the peptide product. Recently, a phase Ib clinical trial was completed, demonstrating a favorable side effect profile of myelin antigen and aquaporine-4 antigen-loaded tolerance-inducing DC (tolDC) for the treatment of a mixed group of MS and neuromyelitis optica patients (50). Similarly, 2 phase I clinical trials using vitamin D3-treated tolerance-inducing DC (tolDC) loaded with a pool of myelin peptides are ongoing [NCT02618902 and NCT02903537 (51)]. These trials were initiated following promising results in a preclinical setting, with $MOG_{40–55}$-loaded vitamin D3-treated murine tolDC showing a beneficial effect on the clinical course of EAE (52, 53).

Myelin-Specific T Cell Vaccination

Deletion of myelin-specific T cells can be aimed for by infusion of autologous anti-myelin T cells attenuated by irradiation. By exposure of the immune system to the self-antigens carried by these attenuated T cells, a T cell response leading to deletion or downregulation of autoreactive T cells is induced (54–57). This so-called myelin-specific T cell vaccination was the subject of several open-label clinical trials, followed by a first double-blind, placebo-controlled clinical trial in 2012. In this trial, 17 relapsing progressive MS patients were treated with a mixture of autologous irradiated T cells reactive to nine different myelin-derived peptides, compared to 7 placebo-treated patients (57). In the T cell-treated group, a significant reduction in Expanded Disability Status Scale (EDSS) score 1 year after treatment could be demonstrated in comparison to an increased score in the placebo-treated group, as well as a reduced relapse rate in the T cell-vaccinated group.

DNA Vaccination

Safety of and immune modulation by BHT-3009, a MBP-encoding DNA plasmid, was evaluated in a phase I/II clinical trial in 30 RR-MS and SP-MS patients and was demonstrated to be safe and well tolerated (58). Antigen-specific immune responses were evaluated in a subgroup of patients, demonstrating a significant decrease in myelin-specific proliferation of IFN-γ-producing CD4+ T cells at week 9 and 50 following BHT-3009 administration in all patients who displayed myelin-reactivity at baseline. Moreover, myelin-specific antibody titers were reduced in the CSF, pointing toward downregulation of myelin-specific immune responses both in the periphery and the central nervous system. Interestingly, tolerance induction was not only confined to MBP but spread to other myelin proteins, both in the CSF and in the peripheral blood.

In a larger phase II clinical trial, 289 RR-MS patients were randomized into three treatment groups comparing placebo, 0.5 mg BHT-3009, and 1.5 mg BHT-3009 (59). Administration was performed intramuscularly at week 0, 2, and 4, followed by 4-weekly administrations until week 44. Treatment with 0.5 mg of BHT-3009 led to a significant reduction in volume of enhancing lesions (51% reduction, p=0.02).

OVERCOMING CHALLENGES OF CURRENT ANTIGEN-SPECIFIC TREATMENT APPROACHES

Although promising results have been achieved with various of the above-mentioned approaches to induce antigen-specific tolerance in MS, several challenges remain (**Table 2**). It is currently generally accepted that myelin-derived proteins are the main antigens targeted by autoreactive responses in MS (1). Nonetheless, the wide variety of MS-associated myelin-derived antigens imposes difficulties for the selection of target antigens for antigen-specific therapies. Additionally, there is a high patient-to-patient variability in myelin reactivity responses (60, 61). Moreover, these responses are often dynamic in time, characterized by loss of tolerance against additional endogenous antigens released during an inflammatory or auto-immune exacerbation. This process is also known as epitope spreading. These newly released epitopes are secondary and differ from the dominant epitopes, toward which the initial autoimmune response was targeted (62). Both intramolecular spreading, *i.e.*, development of autoreactivity against new epitopes of the initial targeted protein, and intermolecular spreading, *i.e.*, spread of autoreactivity to other myelin-derived proteins, have been described (63). Additionally, as demonstrated in the clinical trials focusing on APL, unwanted immune responses following myelin tolerization strategies—both disease exacerbations by augmentation of the targeted Th1/Th17 immune response and hypersensitivity reactions by cytokine shift to a Th2 response—remain a matter of concern. Finally, various questions remain in the light of further clinical translation of antigen-specific therapy, including optimal antigen dose and patient stratification in order to select patients likely to benefit from a specific antigen-specific treatment approach. In the following section, we will discuss different approaches to tackle these challenges.

Lack of Target Antigen Identification, Multi-Epitope Antigen Target and Epitope Spreading

Full-Length Protein Administration by Use of Viral Vectors or Nucleic Acids

Although still requiring knowledge of the target proteins, the use of viral vectors or nucleic acids encoding full-length myelin proteins eliminates the need for prior selection of immune-dominant epitopes, which is in line with the first attempts to induce tolerance in MS using a MBP-encoding DNA vaccine (58, 59). Indeed, following translation of full-length protein encoded by viral vectors or nucleic acids, processing by APC will ensure presentation of a wide variety of naturally processed myelin peptides in a HLA-independent manner.

Viral vector transfection is a versatile method to genetically modify several cell types, including bone marrow cells or differentiated effector cells, to constitutively express myelin proteins. Historically, the use of second-generation viral vectors, such as self-inactivating lentiviral and retroviral vectors, has reduced some of the risks related to vector-based gene therapy such as insertional mutagenesis (64). This has greatly increased the translational potential of this treatment approach. In this context, several preclinical studies demonstrated successful prevention of EAE development following treatment with bone marrow, B or T cells transfected with full-length MOG-encoding retroviral (65–70) or lentiviral (71–73) vectors, as well as with vectors encoding MBP (58, 74–76) or PLP (77). However, to our knowledge, no clinical trials in MS patients using viral vectors are yet planned.

In addition to the use of nuclide acid vaccination with DNA (58, 59), the use of mRNA is gaining interest as well, given its high clinical safety profile because of the transient expression of mRNA and its inability for host genome integration (78, 79). Although direct administration of mRNA has not been investigated in the EAE model, mRNA transfection of carrier cells to induce myelin-derived antigen presentation has been attempted. Indeed, a clinical benefit of treatment with *MOG* mRNA-electroporated tolerogenic DC (tolDC), carrying a wide spectrum of naturally processed MOG-derived epitopes, was recently demonstrated in MOG_{35-55} EAE mice (80). This protective effect was accompanied by a decrease in the MOG_{35-55}-specific pro-inflammatory response in the peripheral immune system and was likely driven by suppression of central nervous system inflammation.

Use of Multi-Epitope Fusion Proteins

Tackling of complex multi-targeted myelin reactivity which is dynamic over time—as is the case for MS—can hypothetically be achieved by broad tolerization with a mix of myelin-derived peptides, as has already been attempted in several of the clinical trials described above, however with varying success. Ideally, antigen-specific therapy should tackle all disease-related autoreactive responses concomittantly in order to downregulate pathogenic myelin reactivity. In addition to further expanding the number of peptides in the peptide mix product, the use of artificial

TABLE 2 | Challenges for next-generation antigen-specific treatment approaches for multiple sclerosis (MS).

Challenge	Possible solution	Treatment approach
Lack of target antigen identification, multi-epitope antigen target and epitope spreading	Use of multiprotein and multi-epitope tolerizing strategies to induce tolerance toward a wide variety of full-length proteins	Nucleic acids, viral vectors, fusion products, peptide mixes
Prevention of unwanted immune responses	Targeting of antigen expression to specific cell populations	Viral vectors, fusion products
	Modification of antigen-specific T cell responses	Fusion products, nanoparticles
Determination of optimal antigen dose for tolerance induction	More insight into low-zone tolerance induction, optimal antigen formulation	
Patient stratification	More insight into parameters for selection of patients likely to benefit from antigen-specific treatment approach	

multi-epitope fusion proteins may be a next step forward in the field of peptide-instigated tolerance induction, since they have been demonstrated to be superior to myelin peptides mixes in preventing or downregulating EAE (81). Indeed, a globular protein product of a synthetic gene encoding different MS-associated epitopes of MBP, PLP, MOG, myelin-associated oligodendrocyte basic protein and oligodendrocyte-specific protein (designated Y-MSPc), displayed stronger capacity to induce T cell anergy, a cytokine shift, and Treg induction when compared to a similar peptide mix, resulting in more effective suppression and even reversal of EAE (81). Although the mode of action behind this stronger immunomodulatory effect by the artificial protein product remains elusive, the authors suggest multiple mechanisms, including lower degradation and clearance rate, more efficient *in vivo* uptake of Y-MSPc, different pathways of MHC-class II presentation (81) and—more recently demonstrated—induction of a specific subset of tolerogenic myeloid CD11c+CD11b+Gr1+ DC (82).

Other examples of tolerance induction in EAE using multi-epitope fusion proteins are readily available. For instance, Elliot et al. generated a fusion protein (MP4), containing full-length MBP and the three hydrophilic domains of PLP (83). Treatment of SJL/J mice with MP4 after EAE induction completely suppressed EAE development, even when EAE induction was performed using adoptive transfer of both MBP- and PLP-reactive T cells (83). Similarly, Zhong et al. demonstrated a strong preventive and therapeutic effect on EAE of a fusion protein containing encephalitogenic epitopes of MBP, MOG, and PLP (84). Interestingly, not only $PLP_{139–151}$-induced EAE was suppressed following intraperitoneal or intravenous administration of the fusion peptide, but also EAE passively induced by T cells reactive against different myelin peptides, demonstrating the ability of the fusion protein to tackle multi-targeted myelin reactivity.

Prevention of Unwanted Immune Responses

Modification of Antigen-Specific T Cell Responses

Direct influence on the T cell response following antigen recognition can be achieved by interference with the T cell-APC interaction or by creation of a tolerogenic environment for antigen presentation, either by fusion of the antigen to tolerizing factors or by antigen presentation using micro- or nanoparticles.

T cells require three signals for full antigen-specific stimulation, *i.e.*, i) interaction of the TCR with MHC-bound antigen on the APC surface, ii) triggering of T-cell bound CD28 by costimulatory molecules CD80 and CD86, and iii) the presence of polarizing cytokines (85). Fusion of disease-specific antigens to molecules involved in this T cell-APC interaction could hypothetically result in tolerance induction by means of antigen presentation while blocking costimulatory signals. In this context, Northrup et al. generated fusion products of $PLP_{139–151}$ with B7 pathway-targeting peptides mimicking CD28 and CTLA-4. This fusion protein interferes with the interaction with costimulatory molecules CD80 and CD86 (86). Subcutaneous administration of the fusion proteins at day 4, 7, and 10 post-EAE induction reduced EAE severity and suppressed weight loss. A cytokine shift was observed, with reduced splenocyte expression of pro-inflammatory IL-2 and GM-CSF, albeit dependent on the particular peptide that was used (86). To the same extent, bifunctional peptide inhibitors (BPI) have been developed to modify T cell responses. BPI consist of antigenic peptides conjugated to adhesion peptides, binding respectively to MHC and costimulatory or adhesion molecules on APC. Binding of a BPI hampers translocation and segregation of the MHC/TCR and costimulatory molecule complexes, preventing the formation of immunological synapse and subsequent T cell activation (87, 88). For instance, Kobayashi et al. demonstrated that linking of $PLP_{139–151}$ to $CD11a_{237–246}$, an intercellular adhesion molecule (ICAM)-1-binding peptide, suppresses PLP-induced EAE severity and incidence. The linked peptide was more effective when compared to a mixture of $PLP_{139–151}$ and $CD11a_{237–246}$ peptides (87). To broaden the antigen-specific immune modulation, thereby tackling epitope spreading, Badawi et al. generated a bivalent BPI consisting of both $MOG_{38–50}$ and $PLP_{139–151}$ bound to an adhesion molecule. In doing so, both $MOG_{38–50}$- and $PLP_{139–151}$-induced EAE was suppressed (88). In the same animal models, the bivalent BPI was superior to $MOG_{38–50}$-BPI and $PLP_{139–151}$-BPI alone for the induction of tolerance (88).

Fusion of myelin epitopes to cytokines or other active compounds by covalent binding can be used to skew the antigen-specific response toward a more tolerogenic profile. Binding of the fused cytokine to receptors on APC leads to specific targeting of the neuroantigen to these APC and enhanced antigen presentation (89). Neuroantigen-fusion proteins with granulocyte macrophage-colony stimulating factor (GM-CSF), being a major cytokine involved in development and differentiation of myeloid APC (90), displayed a more than 1000-fold increase in antigen targeting to APC compared to neuroantigen alone (91). Accordingly, subcutaneous administration of GM-CSF-neuroantigen fusion proteins has shown to be effective in the prevention and treatment of $MOG_{35–55}$- (92, 93), $PLP_{139–151}$- (92, 93) and $MBP_{69–87}$ (89, 91)-induced EAE. Similarly, fusion proteins of myelin proteins with IFN-β (89, 94), IL-16 (89, 95), IL-13 (89, 95), IL-10 (95), IL-2 (89, 95, 96), IL-4 (89), and IL-1RA (89, 95) have been tested in Lewis rat or SJL mice EAE models. Of these, IFN-β and IL-16 gave the highest tolerogenic capacity, however still less effective than GM-CSF (89). In all settings, cytokine-neuroantigen fusion proteins were superior in terms of inhibitory capacity over neuroantigen alone (89, 95), which underlines the benefit of antigen targeting to APC.

Targeting of Antigen Expression to Specific Cell Populations

Targeting of myelin expression to specific cells can enhance tolerance induction and reduce off-target effects by specifically guiding the myelin presentation to possibly tolerogenic environments. For instance, following viral transfection, ubiquitous myelin expression can be prevented by targeting specific cell lineages by using vectors in which expression is under the transcriptional control of specific cell-type promotors. Cell lineages of interest include DC (70–72), as major APC controlling the balance

TABLE 3 | Preclinical evaluation of viral vector transfection, targeting specific cell types, for tolerance induction in EAE.

Author and year	Protein or peptide encoded	Administration approach	Cell type targeted	Animal model	Clinical setting	Results and mode of action
de Andrade Pereira et al. 2013 (71)	Full-length mouse MOG in SIN lentiviral vector	IV transfer of transduced HSC into irradiated C57BL/6 mice	DC by use of DC-STAMP promotor	MOG_{35-55}-induced EAE in C57BL/6 mice	Preventive (EAE induction 8 weeks after HSC transfer)	Full protection by deletion of MOG-specific T cells and generation of Treg
de Andrade Pereira et al. 2015 (72)	Full-length mouse MOG in SIN lentiviral vector	Transfer of transduced BM cells into irradiated C57BL/6 mice	DC by use of DC-STAMP promotor	Passive transfer of 2D2 T cells into C57BL/6 mice	Preventive (transfer 8 weeks before passive EAE induction)	Full protection by induction of unresponsiveness of preactivated MOG-specific CD4+ 2D2 T cells to MOG and acquisition of an anergic or regulatory phenotype by transferred cells
Eixarch et al. 2009 (98)	MOG_{40-55} into Ii molecule in retroviral vector	IV transfer of transduced BM cells into C57BL/6, either partially myeloablated or not myeloablated	MHC class II targeting by replacement of the CLIP-encoding region of the murine Ii molecule by MOG_{40-55}	MOG_{40-55}-induced EAE in C57BL/6 mice	Preventive (transfer 21 days before EAE induction) or therapeutic (transfer 15–17 days after EAE induction)	Protection from EAE development in preventive setting, amelioration of clinical score in therapeutic setting, with increase in IL-5 and IL-10 secretion by splenocytes, pointing towards involvement of Treg
Fransson et al. 2012 (73)	CARαMOG-FoxP3 construct in lentiviral vector	Intranasal transfer of transduced T cells into C57BL/6 mice	CD4+ T cells by direct transfection, Foxp3 driving Treg differentiation	MOG_{35-55}-induced EAE in C57BL/6 mice	Therapeutic (transfer approximately at day 15 after EAE induction, at clinical score of 3)	Reduction of disease symptoms and protection from EAE rechallenge, with reduction of mRNA expression of IFN-γ and IL-12 in the CNS
Keeler et al. 2017 (97)	Full-length MOG in adenovirus-associated vector	IV administration of vector into C57BL/6 mice	Hepatocytes by use of hepatocyte-specific promoter	MOG_{35-55}-induced EAE in C57BL/6 mice	Preventive (transfer 2 weeks before EAE induction) and therapeutic (at different clinical scores)	Protection from EAE development in preventive setting, reversal of mild-to-moderate clinical symptoms in therapeutic setting, reversal of severe clinical symptoms in combination with rapamycine in therapeutic setting, by induction of MOG-specific Treg
Ko et al. 2011 (70)	Full-length mouse MOG in SIN retroviral vector	IV transfer of transduced BM cells into irradiated C57BL/6 mice	DC by use of CD11c promotor	MOG_{35-55}-induced EAE in C57BL/6 mice	Preventive (transfer 8–9 weeks before EAE induction)	Delay in EAE development, but no protection, no mechanistical analyses were performed
Luth et al. 2008 (74)	MBP splice variant in type 5 adenoviral vector	IV administration of vector into FVB mice	Hepatocytes by use of type 5 adenoviral vector	MBP_{1-9}-induced EAE in FVB mice	Preventive (transfer 2 weeks before EAE induction)	Protection from EAE development, by induction of MBP-specific Treg by TGF-β-driven conversion from conventional CD4+CD25– T cells

IV, intravenous; HSC, hematopoietic stem cell; DC, dendritic cell; DC-STAMP, dendritic cell-specific transmembrane protein; Treg, regulatory T cell; SIN, self-inactivating; BM, bone marrow; MHC, major histocompatibility complex; IFN, interferon; IL, interleukin; TGF, transforming growth factor.

between tolerance and immunity, and hepatocytes (74, 97), being part of the tolerogenic environment of the liver. Several viral vector-based cell-targeting treatment approaches have been attempted in the EAE model, which are described in **Table 3**.

Additionally, fusion proteins can be used for direct targeting. Ring et al. generated a fusion protein of $MOG_{35–55}$ and single-chain fragment variables (scFv) specific for DEC205, which is a receptor almost exclusively expressed by DC (99). Injection of this fusion protein was shown to be beneficial for both EAE development and progression when mice were treated before (preventively) or after (therapeutically) disease induction, respectively (99). $MOG_{35–55}$ expression was targeted to DC, which led to significantly reduced levels of TGF-β secretion by DC and increased numbers of IL-10-producing Treg in the spleen (99). Similarly, a fusion product of $MOG_{35–55}$ and anti-Siglec-H antibodies targeted MOG expression to plasmacytoid DC (pDC) and delayed or decreased clinical signs of EAE when administered in a preventive setting or therapeutic setting, respectively (100).

Micro- and Nanoparticle-Based Systems

Following the success of antigen-coupled cell therapy, micro- and nanoparticles were developed as a delivery vehicle for autoantigens, circumventing the need for autologous blood cells, thereby enhancing clinical translation (101). Micro- and nanoparticles can be used as antigen-delivering vehicles that prevent unwanted immune responses using the strategies mentioned above. Indeed, as reviewed by Kishimoto et al., three strategies can be used for tolerance induction using nanoparticles (102). First of all, nanoparticles can make use of natural tolerance processes, such as antigen presentation without costimulation, oral tolerance, or delivery to the tolerogenic liver environment. For instance, Carambia et al. demonstrated a clinical improvement in EAE mice following a single dose of autoantigen-loaded nanoparticles, specifically targeting to liver sinusoidal endothelial cells, associated with a significant higher frequency of Treg in the spleen of nanoparticle-treated mice compared to vehicle-treated mice (103). Secondly, nanoparticles can be used to specifically target tolerogenic receptors. As an example, a nanoparticle containing $MOG_{35–55}$ and a plasmid containing the murine B and T lymphocyte attenuator (BTLA) was created by Yuan et al. (104). Following transfection of DC with this plasmid and subsequent administration of these transfected DC prior to induction of $MOG_{35–55}$ EAE, EAE development could be prevented and was accompanied by an increased frequency of Treg (104). A final approach is to use nanoparticles to co-administer autoantigens together with tolerogenic pharmacological agents, which has been used in the context of EAE in combination with rapamycine (105, 106) and dexamethasone (107). In conclusion, micro- and nanoparticles have been shown to be a versatile treatment modality in preclinical setting, yet no clinical trials are ongoing currently.

Determination of Optimal Antigen Dose

Auto-antigen dose is often extrapolated from dosing from animal models or determined by safety studies, in which the maximal tolerable dose is considered to be the dose of choice. However, the concept of low-zone tolerance, in which low antigen doses are superior in inducing tolerance compared to high doses, has already been known for several decades (108–110). Indeed, also in the context of MS, Garren et al. demonstrated in their phase II clinical trial with the DNA vaccine BHT-3009 that the 0.5 mg group was significantly superior in inducing tolerance compared to the 1.5 mg group, as demonstrated by MRI measures and *in vitro* PLP reactivity (59). Similarly, as demonstrated by Kappos et al. in their phase II clinical trial using an APL derived from $MBP_{83–99}$, a significant decrease in the volume and number of Gd-enhancing lesions could only be detected in the patient group treated with the lowest dose (42). On the other hand, high-zone tolerance has been demonstrated for tolerance induction in other autoimmune diseases, including hemophilia (111), leaving the efficacy of low-zone *versus* high-zone tolerance to be determined for every tolerance-inducing strategy on an individual base. In conclusion, determination of optimal dosing should be based on both tolerability and efficacy.

In addition, optimization of the antigen product formulation to ensure sufficient antigen delivery is warranted for each particular route of administration, since delivery of an appropriate dose of the auto-antigen to the site of interest is of crucial importance for the effective induction of tolerance. For instance, upon oral administration of peptides, passage of low-dose antigen through the gut-associated lymphoid tissue (GALT) induces antigen-specific regulatory T cells (Treg) in the Peyer's patches (22). However, suppression of ongoing autoimmune reactions, as needed in a therapeutic setting, requires large amounts of oral antigen intake, limiting the clinical applicability of this technique (112, 113). Therefore, generation of fusion proteins with higher efficacy should be aimed for, in which the antigen is either directly targeted to the GALT, e.g., by fusion to cholera toxin subunits (114, 115), or in which higher presentation efficacy can be achieved by fusion to cell membrane-associated proteins (24). Similarly, repeated nasal administration of a fusion protein consisting of cholera toxin subunit B and $PLP_{139–151}$ hampered full EAE development (114). Hence, also for the nasal route of administration, formulation of the auto-antigen should be optimized.

Patient Stratification

Selection of patients likely to benefit from a particular antigen-specific therapy would aid in the development of patient-tailored therapies. Based on subgroup analyses, the HLA-DR haplotype has been demonstrated to be a parameter of importance in the immunological and clinical response to the induction of myelin-specific tolerance. This is not surprising, giving the role of APC-bound HLA-DR in the antigen presentation to CD4+ T cells. The importance of the HLA-DR haploptype is especially the case for antigen-specific tolerance induction strategies using peptides, given that some myelin peptides are HLA-restricted (116, 117), meaning that they are preferentially presented by specific HLA-molecules. However, clinical trials using HLA-DR haplotype as an inclusion parameter have yielded conflicting results. This is most likely due to confounding by other parameters, which should be taken into account for patient selection as well. This includes among others the presence of pre-treatment reactivity toward the epitopes contained in the antigen-specific therapy. Although of

major importance in order to be able to assess antigen-specific immune modulation following treatment, pre-treatment myelin-specific reactivity has not been consistently determined in previously conducted clinical trials, limiting the comparative evaluation of the treatment effect on an immunological level.

CONCLUSION

Numerous attempts to restore tolerance toward myelin-derived antigens have been made over the past decades, both in animal models of MS and in clinical trials for MS patients. Many of these treatment approaches have shown to be safe and well-tolerated in phase I/II clinical trials, although results regarding efficacy have appeared to be less unequivocal. Given the complexity of the myelin response to be down-regulated, patient selection in terms of HLA haplotype, myelin reactivity, and previous treatment profile is warranted. This would allow efficacy analysis in a more homogeneous patient population and may guide us in the selection of patients who may potentially benefit from a particular treatment. Indeed, a one-treatment-fits-all approach is unlikely to be successful in the field of antigen-specific therapy for MS, underlying the need for more insight into parameters for patient stratification.

Additionally, current preclinical research is providing new approaches to tackle some of the challenges faced by the currently used approaches, including epitope spreading and unwanted immune responses following myelin tolerization attempts. These new findings should altogether allow to modify currently used antigen-specific approaches with the aim to enhance their clinical efficacy.

In conclusion, several decades of research into antigen-specific therapy for MS has yielded promising results and findings from currently ongoing preclinical work may add to the efficacy of this type of treatment. Ultimately, antigen-specific therapy for MS may lead to a more effective therapy for MS by induction of tolerance to a wide range of myelin-derived antigens without hampering the normal surveillance and effector function of the immune system.

AUTHOR CONTRIBUTIONS

Conceptualization, JD and NC. Writing—original draft, JD. Writing—review and editing, JD, PC, ZB, and NC. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Hohlfeld R, Dornmair K, Meinl E, Wekerle H. The search for the target antigens of multiple sclerosis, part 1: autoreactive CD4+ T lymphocytes as pathogenic effectors and therapeutic targets. *Lancet Neurol* (2015) 15:198–209. doi: 10.1016/S1474-4422(15)00334-8
2. Hemmer B, Kerschensteiner M, Korn T. Role of the innate and adaptive immune responses in the course of multiple sclerosis. *Lancet Neurol* (2015) 14(4):406–19. doi: 10.1016/S1474-4422(14)70305-9
3. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol* (2010) 162(1):1–11. doi: 10.1111/j.1365-2249.2010.04143.x
4. Hohlfeld R, Dornmair K, Meinl E, Wekerle H. The search for the target antigens of multiple sclerosis, part 2: CD8+ T cells, B cells, and antibodies in the focus of reverse-translational research. *Lancet Neurol* (2016) 15(3):317–31. doi: 10.1016/S1474-4422(15)00313-0
5. Ben-Nun A, Kaushansky N, Kawakami N, Krishnamoorthy G, Berer K, Liblau R, et al. From classic to spontaneous and humanized models of multiple sclerosis: impact on understanding pathogenesis and drug development. *J Autoimmun* (2014) 54:33–50. doi: 10.1016/j.jaut.2014.06.004
6. Robinson AP, Harp CT, Noronha A, Miller SD. The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment. *Handb Clin Neurol* (2014) 122:173–89. doi: 10.1016/B978-0-444-52001-2.00008-X
7. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: A comprehensive review. *J Autoimmun* (2015) 64:13–25. doi: 10.1016/j.jaut.2015.06.010
8. Hirsch DL, Ponda P. Antigen-based immunotherapy for autoimmune disease: current status. *Immunotargets Ther* (2015) 4:1–11. doi: 10.2147/ITT.S49656
9. Sabatos-Peyton CA, Verhagen J, Wraith DC. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Curr Opin Immunol* (2010) 22(5):609–15. doi: 10.1016/j.coi.2010.08.006
10. Sundstedt A, O'Neill EJ, Nicolson KS, Wraith DC. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *J Immunol* (2003) 170(3):1240–8. doi: 10.4049/jimmunol.170.3.1240
11. Dersh D, Yewdell JW. I've got algorithm: predicting tumor and autoimmune peptide targets for CD8+ T cells. *J Clin Invest* (2016) 126(12):4399–401. doi: 10.1172/JCI91302
12. Vita R, Overton JA, Greenbaum JA, Ponomarenko J, Clark JD, Cantrell JR, et al. The immune epitope database (IEDB) 3.0. *Nucleic Acids Res* (2015) 43 (Database issue):D405–12. doi: 10.1093/nar/gku938
13. Schrempf W, Ziemssen T. Glatiramer acetate: mechanisms of action in multiple sclerosis. *Autoimmun Rev* (2007) 6(7):469–75. doi: 10.1016/j.autrev.2007.02.003
14. Weiner HL, Mackin GA, Matsui M, Orav EJ, Khoury SJ, Dawson DM, et al. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science* (1993) 259(5099):1321–4. doi: 10.1126/science.7680493
15. Goodkin DE, Shulman M, Winkelhake J, Waubant E, Andersson P, Stewart T, et al. A phase I trial of solubilized DR2:MBP84-102 (AG284) in multiple sclerosis. *Neurology* (2000) 54(7):1414–20. doi: 10.1212/WNL.54.7.1414
16. Warren KG, Catz I, Ferenczi LZ, Krantz MJ. Intravenous synthetic peptide MBP8298 delayed disease progression in an HLA Class II-defined cohort of patients with progressive multiple sclerosis: results of a 24-month double-blind placebo-controlled clinical trial and 5 years of follow-up treatment. *Eur J Neurol* (2006) 13(8):887–95. doi: 10.1111/j.1468-1331.2006.01533.x
17. Freedman MS, Bar-Or A, Oger J, Traboulsee A, Patry D, Young C, et al. A phase III study evaluating the efficacy and safety of MBP8298 in secondary progressive MS. *Neurology* (2011) 77(16):1551–60. doi: 10.1212/WNL.0b013e318233b240
18. Yadav V, Bourdette DN, Bowen JD, Lynch SG, Mattson D, Preiningerova J, et al. Recombinant T-cell receptor ligand (RTL) for treatment of multiple sclerosis: a double-blind, placebo-controlled, phase 1, dose-escalation study. *Autoimmune Dis* (2012) 2012:954739. doi: 10.1155/2012/954739
19. Walczak A, Siger M, Ciach A, Szczepanik M, Selmaj K. Transdermal application of myelin peptides in multiple sclerosis treatment. *JAMA Neurol* (2013) 70(9):1105–9. doi: 10.1001/jamaneurol.2013.3022
20. Streeter HB, Rigden R, Martin KF, Scolding NJ, Wraith DC. Preclinical development and first-in-human study of ATX-MS-1467 for immunotherapy of MS. *Neurol Neuroimmunol Neuroinflamm* (2015) 2(3): e93. doi: 10.1212/NXI.0000000000000093
21. Chataway J, Martin K, Barrell K, Sharrack B, Stolt P, Wraith DC. Effects of ATX-MS-1467 immunotherapy over 16 weeks in relapsing multiple sclerosis. *Neurology* (2018) 2:e93. doi: 10.1212/WNL.0000000000005118
22. Hohol MJ, Khoury SJ, Cook SL, Orav EJ, Hafler DA, Weiner HL. Three-year open protocol continuation study of oral tolerization with myelin antigens in multiple sclerosis and design of a phase III pivotal trial. *Ann N Y Acad Sci* (1996) 778:243–50. doi: 10.1111/j.1749-6632.1996.tb21132.x

23. Anderton SM, Viner NJ, Matharu P, Lowrey PA, Wraith DC. Influence of a dominant cryptic epitope on autoimmune T cell tolerance. *Nat Immunol* (2002) 3(2):175–81. doi: 10.1038/ni756
24. Buerth C, Mausberg AK, Heininger MK, Hartung HP, Kieseier BC, Ernst JF. Oral tolerance induction in experimental autoimmune encephalomyelitis with Candida utilis expressing the immunogenic MOG35-55 peptide. *PloS One* (2016) 11(5):e0155082. doi: 10.1371/journal.pone.0155082
25. Jurynczyk M, Walczak A, Jurewicz A, Jesionek-Kupnicka D, Szczepanik M, Selmaj K. Immune regulation of multiple sclerosis by transdermally applied myelin peptides. *Ann Neurol* (2010) 68(5):593–601. doi: 10.1002/ana.22219
26. Fukaura H, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Hafler DA. Antigen-specific TGF-beta1 secretion with bovine myelin oral tolerization in multiple sclerosis. *Ann N Y Acad Sci* (1996) 778:251–7. doi: 10.1111/j.1749-6632.1996.tb21133.x
27. Fukaura H, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Hafler DA. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J Clin Invest* (1996) 98(1):70–7. doi: 10.1172/JCI118779
28. Hafler DA, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Fukaura H. Oral administration of myelin induces antigen-specific TGF-beta 1 secreting T cells in patients with multiple sclerosis. *Ann N Y Acad Sci* (1997) 835:120–31. doi: 10.1111/j.1749-6632.1997.tb48623.x
29. Bourdette DN, Edmonds E, Smith C, Bowen JD, Guttmann CR, Nagy ZP, et al. A highly immunogenic trivalent T cell receptor peptide vaccine for multiple sclerosis. *Mult Scler* (2005) 11(5):552–61. doi: 10.1191/1352458505ms1225oa
30. Vandenbark AA, Culbertson NE, Bartholomew RM, Huan J, Agotsch M, LaTocha D, et al. Therapeutic vaccination with a trivalent T-cell receptor (TCR) peptide vaccine restores deficient FoxP3 expression and TCR recognition in subjects with multiple sclerosis. *Immunology* (2008) 123 (1):66–78. doi: 10.1111/j.1365-2567.2007.02703.x
31. Vandenbark AA. TCR peptide vaccination in multiple sclerosis: boosting a deficient natural regulatory network that may involve TCR-specific CD4 +CD25+ Treg cells. *Curr Drug Targets Inflammation Allergy* (2005) 4 (2):217–29. doi: 10.2174/1568010053586327
32. Gaur A, Boehme SA, Chalmers D, Crowe PD, Pahuja A, Ling N, et al. Amelioration of relapsing experimental autoimmune encephalomyelitis with altered myelin basic protein peptides involves different cellular mechanisms. *J Neuroimmunol* (1997) 74(1-2):149–58. doi: 10.1016/S0165-5728(96)00220-2
33. Karin N, Mitchell DJ, Brocke S, Ling N, Steinman L. Reversal of experimental autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon gamma and tumor necrosis factor alpha production. *J Exp Med* (1994) 180(6):2227–37. doi: 10.1084/jem.180.6.2227
34. Sakai K, Zamvil SS, Mitchell DJ, Hodgkinson S, Rothbard JB, Steinman L. Prevention of experimental encephalomyelitis with peptides that block interaction of T cells with major histocompatibility complex proteins. *Proc Natl Acad Sci USA* (1989) 86(23):9470–4. doi: 10.1073/pnas.86.23.9470
35. Smilek DE, Wraith DC, Hodgkinson S, Dwivedy S, Steinman L, McDevitt HO. A single amino acid change in a myelin basic protein peptide confers the capacity to prevent rather than induce experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* (1991) 88(21):9633–7. doi: 10.1073/pnas.88.21.9633
36. Wraith DC, Smilek DE, Mitchell DJ, Steinman L, McDevitt HO. Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. *Cell* (1989) 59(2):247–55. doi: 10.1016/0092-8674(89)90287-0
37. Wauben MH, Joosten I, Schlief A, van der Zee R, Boog CJ, van Eden W. Inhibition of experimental autoimmune encephalomyelitis by MHC class II binding competitor peptides depends on the relative MHC binding affinity of the disease-inducing peptide. *J Immunol* (1994) 152(8):4211–20.
38. Franco A, Southwood S, Arrhenius T, Kuchroo VK, Grey HM, Sette A, et al. T cell receptor antagonist peptides are highly effective inhibitors of experimental allergic encephalomyelitis. *Eur J Immunol* (1994) 24(4):940–6. doi: 10.1002/eji.1830240424
39. Santambrogio L, Lees MB, Sobel RA. Altered peptide ligand modulation of experimental allergic encephalomyelitis: immune responses within the CNS. *J Neuroimmunol* (1998) 81(1-2):1–13. doi: 10.1016/S0165-5728(97)00138-0
40. Nicholson LB, Greer JM, Sobel RA, Lees MB, Kuchroo VK. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* (1995) 3(4):397–405. doi: 10.1016/1074-7613(95)90169-8
41. Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med* (2000) 6(10):1167–75. doi: 10.1038/80516
42. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. *Altered Pept Ligand Relapsing MS Study Group Nat Med* (2000) 6(10):1176–82. doi: 10.1038/80525
43. Crowe PD, Qin Y, Conlon PJ, Antel JP. NBI-5788, an altered MBP83-99 peptide, induces a T-helper 2-like immune response in multiple sclerosis patients. *Ann Neurol* (2000) 48(5):758–65. doi: 10.1002/1531-8249(200011)48:5<758::AID-ANA9>3.0.CO;2-2
44. Deraos G, Rodi M, Kalbacher H, Chatzantoni K, Karagiannis F, Synodinos L, et al. Properties of myelin altered peptide ligand cyclo(87-99)(Ala91,Ala96) MBP87-99 render it a promising drug lead for immunotherapy of multiple sclerosis. *Eur J Med Chem* (2015) 101:13–23. doi: 10.1016/j.ejmech.2015.06.015
45. Leech MD, Chung CY, Culshaw A, Anderton SM. Peptide-based immunotherapy of experimental autoimmune encephalomyelitis without anaphylaxis. *Eur J Immunol* (2007) 37(12):3576–81. doi: 10.1002/eji.200737148
46. Tselios T, Apostolopoulos V, Daliani I, Deraos S, Grdadolnik S, Mavromoustakos T, et al. Antagonistic effects of human cyclic MBP(87-99) altered peptide ligands in experimental allergic encephalomyelitis and human T-cell proliferation. *J Med Chem* (2002) 45(2):275–83. doi: 10.1021/jm0102147
47. Lutterotti A, Yousef S, Sputtek A, Sturner KH, Stellmann JP, Breiden P, et al. Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. *Sci Transl Med* (2013) 5(188):188ra75. doi: 10.1126/scitranslmed.3006168
48. Turley DM, Miller SD. Peripheral tolerance induction using ethylenecarbodiimide-fixed APCs uses both direct and indirect mechanisms of antigen presentation for prevention of experimental autoimmune encephalomyelitis. *J Immunol* (2007) 178(4):2212–20. doi: 10.4049/jimmunol.178.4.2212
49. Zurich W. *Establishing immune tolerance in multiple sclerosis with peptide-coupled red blood cells*. (2018). Available at: https://www.wysszurich.uzh.ch/projects/wyss-zurich-projects/etimsred.
50. Zubizarreta I, Florez-Grau G, Vila G, Cabezon R, Espana C, Andorra M, et al. Immune tolerance in multiple sclerosis and neuromyelitis optica with peptide-loaded tolerogenic dendritic cells in a phase 1b trial. *Proc Natl Acad Sci USA* (2019) 116(17):8463–70. doi: 10.1073/pnas.1820039116
51. Willekens B, Presas-Rodriguez S, Mansilla MJ, Derdelinckx J, Lee WP, Nijs G, et al. Tolerogenic dendritic cell-based treatment for multiple sclerosis (MS): a harmonised study protocol for two phase I clinical trials comparing intradermal and intranodal cell administration. *BMJ Open* (2019) 9(9): e030309. doi: 10.1136/bmjopen-2019-030309
52. Mansilla MJ, Contreras-Cardone R, Navarro-Barriuso J, Cools N, Berneman Z, Ramo-Tello C, et al. Cryopreserved vitamin D3-tolerogenic dendritic cells pulsed with autoantigens as a potential therapy for multiple sclerosis patients. *J Neuroinflamm* (2016) 13(1):113. doi: 10.1186/s12974-016-0584-9
53. Mansilla MJ, Selles-Moreno C, Fabregas-Puig S, Amoedo J, Navarro-Barriuso J, Teniente-Serra A, et al. Beneficial effect of tolerogenic dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. *CNS Neurosci Ther* (2015) 21(3):222–30. doi: 10.1111/cns.12342
54. Vandenbark AA, Abulafia-Lapid R. Autologous T-cell vaccination for multiple sclerosis: a perspective on progress. *BioDrugs* (2008) 22(4):265–73. doi: 10.2165/00063030-200822040-00006
55. Hellings N, Raus J, Stinissen P. T-cell-based immunotherapy in multiple sclerosis: induction of regulatory immune networks by T-cell vaccination. *Expert Rev Clin Immunol* (2006) 2(5):705–16. doi: 10.1586/1744666X.2.5.705
56. Achiron A, Lavie G, Kishner I, Stern Y, Sarova-Pinhas I, Ben-Aharon T, et al. T cell vaccination in multiple sclerosis relapsing-remitting nonresponders patients. *Clin Immunol* (2004) 113(2):155–60. doi: 10.1016/j.clim.2004.06.004
57. Karussis D, Shor H, Yachnin J, Lanxner N, Amiel M, Baruch K, et al. T cell vaccination benefits relapsing progressive multiple sclerosis patients: a

randomized, double-blind clinical trial. *PloS One* (2012) 7(12):e50478. doi: 10.1371/journal.pone.0050478
58. Bar-Or A, Vollmer T, Antel J, Arnold DL, Bodner CA, Campagnolo D, et al. Induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled phase 1/2 trial. *Arch Neurol* (2007) 64(10):1407–15. doi: 10.1001/archneur.64.10.nct70002
59. Garren H, Robinson WH, Krasulova E, Havrdova E, Nadj C, Selmaj K, et al. Phase 2 trial of a DNA vaccine encoding myelin basic protein for multiple sclerosis. *Ann Neurol* (2008) 63(5):611–20. doi: 10.1002/ana.21370
60. Saez-Torres I, Brieva L, Espejo C, Barrau MA, Montalban X, Martinez-Caceres EM. Specific proliferation towards myelin antigens in patients with multiple sclerosis during a relapse. *Autoimmunity* (2002) 35(1):45–50. doi: 10.1080/08916930290005927
61. Greer JM. Autoimmune T-cell reactivity to myelin proteolipids and glycolipids in multiple sclerosis. *Mult Scler Int* (2013) 2013:151427. doi: 10.1155/2013/151427
62. Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* (2002) 2(2):85–95. doi: 10.1038/nri724
63. Tuohy VK, Yu M, Yin L, Kawczak JA, Johnson JM, Mathisen PM, et al. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Rev* (1998) 164:93–100. doi: 10.1111/j.1600-065X.1998.tb01211.x
64. David RM, Doherty AT. Viral vectors: the road to reducing genotoxicity. *Toxicol Sci* (2017) 155(2):315–25. doi: 10.1093/toxsci/kfw220
65. Chan J, Ban EJ, Chun KH, Wang S, Backstrom BT, Bernard CC, et al. Transplantation of bone marrow transduced to express self-antigen establishes deletional tolerance and permanently remits autoimmune disease. *J Immunol* (2008) 181(11):7571–80. doi: 10.4049/jimmunol.181.11.7571
66. Chen XT, Chan ST, Hosseini H, Layton D, Boyd R, Alderuccio F, et al. Transplantation of retrovirally transduced bone marrow prevents autoimmune disease in aged mice by peripheral tolerance mechanisms. *Autoimmunity* (2011) 44(5):384–93. doi: 10.3109/08916934.2010.541173
67. Chung JY, Figgett W, Fairfax K, Bernard C, Chan J, Toh BH, et al. Gene therapy delivery of myelin oligodendrocyte glycoprotein (MOG) via hematopoietic stem cell transfer induces MOG-specific B cell deletion. *J Immunol* (2014) 192(6):2593–601. doi: 10.4049/jimmunol.1203563
68. Hosseini H, Oh DY, Chan ST, Chen XT, Nasa Z, Yagita H, et al. Non-myeloablative transplantation of bone marrow expressing self-antigen establishes peripheral tolerance and completely prevents autoimmunity in mice. *Gene Ther* (2012) 19(11):1075–84. doi: 10.1038/gt.2011.179
69. Nasa Z, Chung JY, Chan J, Toh BH, Alderuccio F, et al. Nonmyeloablative conditioning generates autoantigen-encoding bone marrow that prevents and cures an experimental autoimmune disease. *Am J Transplant* (2012) 12 (8):2062–71. doi: 10.1111/j.1600-6143.2012.04068.x
70. Ko HJ, Chung JY, Nasa Z, Chan J, Siatskas C, Toh BH, et al. Targeting MOG expression to dendritic cells delays onset of experimental autoimmune disease. *Autoimmunity* (2011) 44(3):177–87. doi: 10.3109/08916934.2010.515274
71. de Andrade Pereira B, Fraefel C, Hilbe M, Ackermann M, Dresch C, et al. Transcriptional targeting of DCs with lentiviral vectors induces antigen-specific tolerance in a mouse model of multiple sclerosis. *Gene Ther* (2013) 20(5):556–66. doi: 10.1038/gt.2012.73
72. de Andrade Pereira B, Ackermann M, Chaudhary S, Vogel R, Vogt B, Dresch C, et al. Tolerance of activated pathogenic CD4+ T cells by transcriptional targeting of dendritic cells. *Gene Ther* (2015) 22(5):382–90. doi: 10.1038/gt.2015.6
73. Fransson M, Piras E, Burman J, Nilsson B, Essand M, Lu B, et al. CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J Neuroinflamm* (2012) 9:112. doi: 10.1186/1742-2094-9-112
74. Luth S, Huber S, Schramm C, Buch T, Zander S, Stadelmann C, et al. Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuroinflammation by inducing antigen-specific Tregs. *J Clin Invest* (2008) 118(10):3403–10. doi: 10.1172/JCI32132
75. Melo ME, Qian J, El-Amine M, Agarwal RK, Soukhareva N, Kang Y, et al. Gene transfer of Ig-fusion proteins into B cells prevents and treats autoimmune diseases. *J Immunol* (2002) 168(9):4788–95. doi: 10.4049/jimmunol.168.9.4788
76. Xu B, Scott DW. A novel retroviral gene therapy approach to inhibit specific antibody production and suppress experimental autoimmune encephalomyelitis induced by MOG and MBP. *Clin Immunol* (2004) 111(1):47–52. doi: 10.1016/j.clim.2003.12.013
77. Xu B, Haviernik P, Wolfraim LA, Bunting KD, Scott DW. Bone marrow transplantation combined with gene therapy to induce antigen-specific tolerance and ameliorate EAE. *Mol Ther* (2006) 13(1):42–8. doi: 10.1016/j.ymthe.2005.09.002
78. Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood* (2001) 98(1):49–56. doi: 10.1182/blood.V98.1.49
79. Van Tendeloo VF, Ponsaerts P, Berneman ZN. mRNA-based gene transfer as a tool for gene and cell therapy. *Curr Opin Mol Ther* (2007) 9(5):423–31.
80. Derdelinckx J, Mansilla MJ, De Laere M, Lee WP, Navarro-Barriuso J, Wens I, et al. Clinical and immunological control of experimental autoimmune encephalomyelitis by tolerogenic dendritic cells loaded with MOG-encoding mRNA. *J Neuroinflamm* (2019) 16(1):167. doi: 10.1186/s12974-019-1541-1
81. Kaushansky N, Kerlero de Rosbo N, Zilkha-Falb R, Yosef-Hemo R, Cohen L, Ben-Nun A. 'Multi-epitope-targeted' immune-specific therapy for a multiple sclerosis-like disease via engineered multi-epitope protein is superior to peptides. *PloS One* (2011) 6(11):e27860. doi: 10.1371/journal.pone.0027860
82. Kaushansky N, Kaminitz A, Allouche-Arnon H, Ben-Nun A. Modulation of MS-like disease by a multi epitope protein is mediated by induction of CD11c(+)CD11b(+)Gr1(+) myeloid-derived dendritic cells. *J Neuroimmunol* (2019) 333:476953. doi: 10.1016/j.jneuroim.2019.04.013
83. Elliott EA, McFarland HI, Nye SH, Cofiell R, Wilson TM, Wilkinset al. JA. Treatment of experimental encephalomyelitis with a novel chimeric fusion protein of myelin basic protein and proteolipid protein. *J Clin Invest* (1996) 98(7):1602–12. doi: 10.1172/JCI118954
84. Zhong MC, Kerlero de Rosbo N, Ben-Nun A. Multiantigen/multiepitope-directed immune-specific suppression of "complex autoimmune encephalomyelitis" by a novel protein product of a synthetic gene. *J Clin Invest* (2002) 110(1):81–90. doi: 10.1172/JCI0215692
85. Corthay A. A three-cell model for activation of naive T helper cells. *Scand J Immunol* (2006) 64(2):93–6. doi: 10.1111/j.1365-3083.2006.01782.x
86. Northrup L, Sestak JO, Sullivan BP, Thati S, Hartwell BL, Siahaan TJ, et al. Co-delivery of autoantigen and b7 pathway modulators suppresses experimental autoimmune encephalomyelitis. *AAPS J* (2014) 16(6):1204–13. doi: 10.1208/s12248-014-9671-y
87. Kobayashi N, Kobayashi H, Gu L, Malefyt T, Siahaan TJ. Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *J Pharmacol Exp Ther* (2007) 322(2):879–86. doi: 10.1124/jpet.107.123257
88. Badawi AH, Siahaan TJ. Suppression of MOG- and PLP-induced experimental autoimmune encephalomyelitis using a novel multivalent bifunctional peptide inhibitor. *J Neuroimmunol* (2013) 263(1-2):20–7. doi: 10.1016/j.jneuroim.2013.07.009
89. Mannie MD, Blanchfield JL, Islam SM, Abbott DJ. Cytokine-neuroantigen fusion proteins as a new class of tolerogenic, therapeutic vaccines for treatment of inflammatory demyelinating disease in rodent models of multiple sclerosis. *Front Immunol* (2012) 3:255. doi: 10.3389/fimmu.2012.00255
90. Conti L, Gessani S. GM-CSF in the generation of dendritic cells from human blood monocyte precursors: recent advances. *Immunobiology* (2008) 213(9-10):859–70. doi: 10.1016/j.imbio.2008.07.017
91. Blanchfield JL, Mannie MD. A GMCSF-neuroantigen fusion protein is a potent tolerogen in experimental autoimmune encephalomyelitis (EAE) that is associated with efficient targeting of neuroantigen to APC. *J Leukoc Biol* (2010) 87(3):509–21. doi: 10.1189/jlb.0709520
92. Abbott DJ, Blanchfield JL, Martinson DA, Russell SC, Taslim N, Curtis AD, et al. Neuroantigen-specific, tolerogenic vaccines: GM-CSF is a fusion partner that facilitates tolerance rather than immunity to dominant self-epitopes of myelin in murine models of experimental autoimmune encephalomyelitis (EAE). *BMC Immunol* (2011) 12:72. doi: 10.1186/1471-2172-12-72
93. Islam SM, Curtis AD2, Taslim N, Wilkinson DS, Mannie MD. GM-CSF-neuroantigen fusion proteins reverse experimental autoimmune encephalomyelitis and mediate tolerogenic activity in adjuvant-primed environments: association with inflammation-dependent, inhibitory

antigen presentation. *J Immunol* (2014) 193(5):2317–29. doi: 10.4049/jimmunol.1303223
94. Mannie MD, Abbott DJ, Blanchfield JL. Experimental autoimmune encephalomyelitis in Lewis rats: IFN-beta acts as a tolerogenic adjuvant for induction of neuroantigen-dependent tolerance. *J Immunol* (2009) 182 (9):5331–41. doi: 10.4049/jimmunol.0803756
95. Mannie MD, Abbott DJ. A fusion protein consisting of IL-16 and the encephalitogenic peptide of myelin basic protein constitutes an antigen-specific tolerogenic vaccine that inhibits experimental autoimmune encephalomyelitis. *J Immunol* (2007) 179(3):1458–65. doi: 10.4049/jimmunol.179.3.1458
96. Mannie MD, Clayson BA, Buskirk EJ, DeVine JL, Hernandez JJ, Abbott DJ. IL-2/neuroantigen fusion proteins as antigen-specific tolerogens in experimental autoimmune encephalomyelitis (EAE): correlation of T cell-mediated antigen presentation and tolerance induction. *J Immunol* (2007) 178(5):2835–43. doi: 10.4049/jimmunol.178.5.2835
97. Keeler GD, Kumar S, Palaschak B, Silverberg EL, Markusic DM, Jones NT, et al. Gene therapy-induced antigen-specific Tregs inhibit neuro-inflammation and reverse disease in a mouse model of multiple sclerosis. *Mol Ther* (2017) 187:6346–56. doi: 10.1016/j.ymthe.2017.09.001
98. Eixarch H, Espejo C, Gomez A, Mansilla MJ, Castillo M, Mildner A, et al. Tolerance induction in experimental autoimmune encephalomyelitis using non-.myeloablative hematopoietic gene therapy with autoantigen. *Mol Ther* (2009) 17(5):897–905. doi: 10.1038/mt.2009.42
99. Ring S, Maas M, Nettelbeck DM, Enk AH, Mahnke K. Targeting of autoantigens to DEC205(+) dendritic cells in vivo suppresses experimental allergic encephalomyelitis in mice. *J Immunol* (2013) 191(6):2938–47. doi: 10.4049/jimmunol.1202592
100. Loschko J, Heink S, Hackl D, Dudziak D, Reindl W, Korn T, et al. Antigen targeting to plasmacytoid dendritic cells via Siglec-H inhibits Th cell-dependent autoimmunity. *J Immunol* (2011) 187(12):6346–56. doi: 10.4049/jimmunol.1102307
101. Quintana FJ. Nanoparticles for the induction of antigen-specific Tregs. *Immunotherapy* (2013) 5(5):437–40. doi: 10.2217/imt.13.25
102. Kishimoto TK, Maldonado RA. Nanoparticles for the induction of antigen-specific immunological tolerance. *Front Immunol* (2018) 9:230. doi: 10.3389/fimmu.2018.00230
103. Carambia A, Freund B, Schwinge D, Bruns OT, Salmen SC, Ittrich H, et al. Nanoparticle-based autoantigen delivery to Treg-inducing liver sinusoidal endothelial cells enables control of autoimmunity in mice. *J Hepatol* (2015) 62(6):1349–56. doi: 10.1016/j.jhep.2015.01.006
104. Yuan B, Zhao L, Fu F, Liu Y, Lin C, Wu X, et al. A novel nanoparticle containing MOG peptide with BTLA induces T cell tolerance and prevents multiple sclerosis. *Mol Immunol* (2014) 57(2):93–9. doi: 10.1016/j.molimm.2013.08.006
105. Maldonado RA, LaMothe RA, Ferrari JD, Zhang AH, Rossi RJ, Kolte PN, et al. Polymeric synthetic nanoparticles for the induction of antigen-specific immunological tolerance. *Proc Natl Acad Sci USA* (2015) 112(2):E156–65. doi: 10.1073/pnas.1408686111
106. Tostanoski LH, Chiu YC, Gammon JM, Simon T, Andorko JI, Bromberg JS, et al. Reprogramming the local lymph node microenvironment promotes tolerance that is systemic and antigen specific. *Cell Rep* (2016) 16(11):2940–52. doi: 10.1016/j.celrep.2016.08.033
107. Peine KJ, Guerau-de-Arellano M, Lee P, Kanthamneni N, Severin M, Probst GD, et al. Treatment of experimental autoimmune encephalomyelitis by codelivery of disease associated Peptide and dexamethasone in acetalated dextran microparticles. *Mol Pharm* (2014) 11(3):828–35. doi: 10.1021/mp4005172
108. Seidel-Guyenot W, Perschon S, Dechant N, Alt R, Knop J, Steinbrink K. Low zone tolerance induced by systemic application of allergens inhibits Tc1-mediated skin inflammation. *J Allergy Clin Immunol* (2006) 117(5):1170–7. doi: 10.1016/j.jaci.2006.01.014
109. Schmidt T, Lorenz N, Raker V, Reissig S, Waisman A, Weigmann B, et al. Epicutaneous and oral low-zone tolerance protects from colitis in mice. *J Invest Dermatol* (2016) 136(9):1831–9. doi: 10.1016/j.jid.2016.04.037
110. Kolsch E, Stumpf R, Weber G. Low zone tolerance and suppressor T cells. *Transplant Rev* (1975) 26:56–86. doi: 10.1111/j.1600-065X.1975.tb00175.x
111. Steinman L, Ho PP, Robinson WH, Utz PJ, Villoslada P. Antigen-specific tolerance to self-antigens in protein replacement therapy, gene therapy and autoimmunity. *Curr Opin Immunol* (2019) 61:46–53. doi: 10.1016/j.coi.2019.07.011
112. Thompson HS, Staines NA. Could specific oral tolerance be a therapy for autoimmune disease? *Immunol Today* (1990) 11(11):396–9. doi: 10.1016/0167-5699(90)90158-6
113. Sun JB, Rask C, Olsson T, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proc Natl Acad Sci USA* (1996) 93(14):7196–201. doi: 10.1073/pnas.93.14.7196
114. Yuki Y, Byun Y, Fujita M, Izutani W, Suzuki T, Udaka S, et al. Production of a recombinant hybrid molecule of cholera toxin-B-subunit and proteolipid-protein-peptide for the treatment of experimental encephalomyelitis. *Biotechnol Bioeng* (2001) 74(1):62–9. doi: 10.1002/bit.1095
115. Kap YS, van Driel N, Arends R, Rouwendal G, Verolin M, Blezer E, et al. Immune modulation by a tolerogenic myelin oligodendrocyte glycoprotein (MOG)10-60 containing fusion protein in the marmoset experimental autoimmune encephalomyelitis model. *Clin Exp Immunol* (2015) 180 (1):28–39. doi: 10.1111/cei.12487
116. Berthelot L, Laplaud DA, Pettre S, Ballet C, Michel L, Hillion S, et al. Blood CD8+ T cell responses against myelin determinants in multiple sclerosis and healthy individuals. *Eur J Immunol* (2008) 38(7):1889–99. doi: 10.1002/eji.200838023
117. Martin R, Jaraquemada D, Flerlage M, Richert J, Whitaker J, Long EO, et al. Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J Immunol* (1990) 145(2):540–8.
118. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* (2004) 199(7):971–9. doi: 10.1084/jem.20031579
119. Libbey JE, McCoy LL, Fujinami RS. Molecular mimicry in multiple sclerosis. *Int Rev Neurobiol* (2007) 79:127–47. doi: 10.1016/S0074-7742(07)79006-2
120. Mallucci G, Peruzzotti-Jametti L, Bernstock JD, Pluchino S. The role of immune cells, glia and neurons in white and gray matter pathology in multiple sclerosis. *Prog Neurobiol* (2015) 127-128:1–22. doi: 10.1016/j.pneurobio.2015.02.003
121. Sospedra M, Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* (2005) 23:683–747. doi: 10.1146/annurev.immunol.23.021704.115707
122. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* (1995) 80(5):695–705. doi: 10.1016/0092-8674(95)90348-8
123. Henderson AP, Barnett MH, Parratt JD, Prineas JW. Multiple sclerosis: distribution of inflammatory cells in newly forming lesions. *Ann Neurol* (2009) 66(6):739–53. doi: 10.1002/ana.21800
124. Traka M, Podojil JR, McCarthy DP, Miller SD, Popko B. Oligodendrocyte death results in immune-mediated CNS demyelination. *Nat Neurosci* (2016) 19(1):65–74. doi: 10.1038/nn.4193
125. Locatelli G, Wortge S, Buch T, Ingold B, Frommer F, Sobottka B, et al. Primary oligodendrocyte death does not elicit anti-CNS immunity. *Nat Neurosci* (2012) 15(4):543–50. doi: 10.1038/nn.3062
126. Nuyts AH, Lee WP, Bashir-Dar R, Berneman ZN, Cools N. Dendritic cells in multiple sclerosis: key players in the immunopathogenesis, key players for new cellular immunotherapies? *Mult Scler* (2013) 19(8):995–1002. doi: 10.1177/1352458512473189

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 10 March 2021
doi: 10.3389/fimmu.2021.633436

Regulatory Dendritic Cells, T Cell Tolerance, and Dendritic Cell Therapy for Immunologic Disease

Sara Ness[1], *Shiming Lin*[1] *and John R. Gordon*[1,2*]

[1] Department of Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada, [2] Division of Respirology, Critical Care and Sleep Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Dendritic cells (DC) are antigen-presenting cells that can communicate with T cells both directly and indirectly, regulating our adaptive immune responses against environmental and self-antigens. Under some microenvironmental conditions DC develop into anti-inflammatory cells which can induce immunologic tolerance. A substantial body of literature has confirmed that in such settings regulatory DC (DCreg) induce T cell tolerance by suppression of effector T cells as well as by induction of regulatory T cells (Treg). Many *in vitro* studies have been undertaken with human DCreg which, as a surrogate marker of antigen-specific tolerogenic potential, only poorly activate allogeneic T cell responses. Fewer studies have addressed the abilities of, or mechanisms by which these human DCreg suppress autologous effector T cell responses and induce infectious tolerance-promoting Treg responses. Moreover, the agents and properties that render DC as tolerogenic are many and varied, as are the cells' relative regulatory activities and mechanisms of action. Herein we review the most current human and, where gaps exist, murine DCreg literature that addresses the cellular and molecular biology of these cells. We also address the clinical relevance of human DCreg, highlighting the outcomes of pre-clinical mouse and non-human primate studies and early phase clinical trials that have been undertaken, as well as the impact of innate immune receptors and symbiotic microbial signaling on the immunobiology of DCreg.

Keywords: tolerance, dendritic cell, regulatory T cell, immunologic disease, mechanism

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Silvia Gregori,
San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Italy
Dalia Raich Regué,
Mar Institute of Medical Research (IMIM), Spain

***Correspondence:**
John R. Gordon
john.gordon@usask.ca

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *25 November 2020*
Accepted: *18 January 2021*
Published: *10 March 2021*

Citation:
Ness S, Lin S and Gordon JR (2021) Regulatory Dendritic Cells, T Cell Tolerance, and Dendritic Cell Therapy for Immunologic Disease. Front. Immunol. 12:633436. doi: 10.3389/fimmu.2021.633436

INTRODUCTION

In the early 1970s, dendritic cells (DC) were discovered and identified as proficient antigen-presenting cells that were capable of potently activating T cells (1–4). By the early 1990s, DC researchers had begun to uncover the regulatory roles of naturally-occurring DC (5), as well as new ways to convert immature DC into tolerance-promoting antigen-presenting cells (6, 7). However, these experiments were limited due to the small numbers of differentiated DC that can be collected from human or mouse tissues (8, 9), such that it was not until methods were developed to differentiate DC *in vitro* that DC research really became a mainstream sub-discipline in immunology.

As noted above, DC develop from bone marrow progenitor cells that complete their differentiation in the periphery. Classically, they were thought to differentiate into either "migratory" or "tissue resident" cells, although later studies exploring their relative gene expression patterns delineated a more specific classification system based primarily on lineage (10–13). There are now considered to be five categories of DC: plasmacytoid DC (pDC); conventional DC (cDC), including cDC1 and cDC2 (the latter are also called myeloid DC [mDC]); monocyte-derived DC (mo-DC); and Langerhans cells (14–17). Each of these has been identified in multiple mammalian species, including mice, humans, non-human primates (NHP), and pigs (10, 12, 18). The anatomic localization, immunologic functions, and expression of surface markers, secreted mediators and Toll-like receptors [TLR(s)] by these different groups of murine and human DC has recently been reviewed (19).

In general, cDC are well suited for extra- and intracellular pathogen recognition and antigen presentation to naïve $CD4^+$ and $CD8^+$ T cells, while pDC are more often associated with protective antiviral and systemic autoimmune responses, a consequence of being activated primarily by TLRs that recognize intracellular viral or self-DNA/RNA species (20–24). Langerhans cells are present in the epidermis and have a role in both tolerance and immune priming in that compartment. Mo-DCs differentiate from monocytes recruited during ongoing tissue inflammatory responses [e.g. (25)] and in turn direct the differentiation of $CD4^+$ T cells to Th1, Th2, or Th17 cells (26, 27). Recent research has revealed that these DC categories are much more fluid than once thought, inasmuch as convergence of differentiation pathways and transitioning between the different types of DC is evident (28–30), making categorization of distinct DC sub-populations an increasingly nebulous concept.

As suggested, in the later 1990s the development of *in vitro* conditions for generating DC from bone marrow and/or peripheral blood progenitors (31, 32) allowed for a surge in research progress. As sentinel antigen-presenting cells DC are well situated immediately adjacent to or integrated into our epithelial cell interfaces with our external environment (e.g., lungs and gut). As steady state cells they are avidly phagocytic, such that they regularly sample their external environment, ingesting and processing all manner of exogenous and endogenous agents, including apoptotic cells, and load the processed antigen peptides onto MHCII molecules for presentation to T cells. DC that are exposed to inflammatory signals during antigen acquisition upregulate their expression of peptide-loaded MHCII, co-stimulatory (e.g., CD40, CD86) and lymph node-homing chemokine receptors, and their expression of inflammatory mediators such as IL-12, while down-regulating their phagocytic activities. They do so while migrating to draining lymph nodes, where they present their processed antigen peptides to T cells in an immunostimulatory context, activating effector T cell (e.g., Th1, Th17) responses (33). If, however, tissue DC acquire otherwise innocuous antigens (e.g., apoptotic cells) in the absence of inflammatory signals, they do not upregulate their antigen-presenting machinery or inflammatory cytokine secretion. As such they provide only low levels of MHCII-bound antigen peptides, co-stimulatory markers and secreted IL-12 signaling to T cells, and thereby induce T cell anergy (34). On the other hand, the DC from mice exposed to innocuous respiratory antigens produce low levels of IL-10 (and less IL-12), while presenting the processed antigens to T cells, and thereby induce Treg responses (35). In the gut, steady state epithelial cells secrete retinoic acid and TGFβ, such that the endogenous gut DC express CD103, TGFβ, and retinoic acid, and thereby induce the differentiation of TGFβ-secreting $CD25^+LAP^+Foxp3^-$ Th3-type Treg (36–38). As DC research became more accessible it was clear that such regulatory DC (DCreg) could be induced under many different conditions, many of which gave rise to DCreg of a unique phenotype, with differing capacities to and mechanisms by which they regulate immune responses (39). Because of this, harnessing the tolerogenic potential of DCreg for the treatment of disease calls for careful consideration of the optimal type of DCreg for each application.

Naturally-occurring and induced DCreg are able to generate robust antigen-specific tolerance by suppressing other immune cells, including Teff, as well as by inducing the differentiation of $CD4^+$ regulatory T cell (Treg) populations. Tolerogenic DC can also directly or indirectly (40) induce regulatory responses among B cells, natural killer cells and $CD8^+$ T cells (41–49), but we will focus herein on DCreg-$CD4^+$ T cell interactions. Specifically, we will review more recent DCreg research, focusing on the studies that have been conducted since our last review of this topic (50). We will address recent progress with human DCreg, but we will also discuss non-human studies where they may shed light on gaps in our knowledge. Furthermore, we will examine the literature that characterizes such DCreg at the cellular and molecular levels and will address the tolerogenic potential and clinical applicability of these cells.

DCreg Suppression of Effector T Cell Responses

DCreg-T cell conversations are facilitated by cell surface molecules and secreted mediators that can directly suppress effector T cell (Teff) responses. We have reported that steady-state $CD8\alpha^+$ splenic DC from mice can suppress Th2 Teff cell responses through their expression of the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO), but also through expression of IL-10 and TGFβ (51). IDO depletes the local cell environment of tryptophan, an essential amino acid for T cells, and thereby activates the Generalized Controller Non-derepressible-2 Kinase (GCN2) (16, 32, 33), a sensor of cellular amino acid levels, leading to T cell apoptosis (52). In addition, kynurenine break-down products of tryptophan, including 3-hydroxyanthranilic and quinolinic acids, can induce a caspase-8-dependent, but Fas-independent apoptosis of T cells (53). Nevertheless, Fas/FasL signaling can also play a role in DCreg suppression of Teff cells. For example, $CD8\alpha^+$ splenic DC (54) and splenic stroma-educated DCreg (55) induce T cell apoptosis in a Fas/FasL-dependent fashion. In the latter case, FasL signaling activates caspases-3 and -8 in T cells to directly

activate apoptosis. However, FasL signaling by DCreg also augments IFNγ secretion by T cells, which in turn induces NO production by the DC, and that further augments $CD4^+$ T effector cell apoptosis (55).

Many reports have addressed the induction of T cell anergy by IL-10-induced human DCreg which, as semi-mature (56) or immature (57) DC (so-called DC10 or DC-10, respectively), express an array of inhibitory receptors (e.g., PD-L1, PD-L2, ILT3, ILT4, HLA-G) (56, 57). It has long been known that HLA-G-expressing antigen-presenting cells can induce T cell anergy (58), but it was more recently recognized that the levels at which HLA-G is expressed by DC-10, at least, correlates with the cell's regulatory activities (59). However, not all inhibitory factors expressed by a DCreg necessarily contribute to the cell's regulatory activities. Thus, vitamin D/IFNγ-induced human DCreg express IL-10, HLA-G, PD-L1 and low levels of FasL, but among these it was only their expression of PD-L1 that was reported as integral to the cell's regulatory activities (60). It is also apparent that inhibitory receptors can play more than one role in immune tolerance. For example, the PD-L1 expressed by TGFβ-induced DCreg is important both to the induction of T cell apoptosis and the differentiation of Treg (61), both of which are essential to successful immunotherapeutic outcomes. Indeed, it has been suggested that the PD-L1:CD86 expression ratio within vitamin D/IL-10-induced DCreg will be a useful predictive marker of the cell's immunotherapeutic efficacy in the clinic (62). Another, less-reported DC inhibitory receptor is CD31, expressed at high levels on GM-CSF-induced mouse bone marrow DC and on human $CD34^+$ stem cell-differentiated DC that have been exposed to VitD. Engagement of $CD31^+$ DC with T cells strongly inhibits their activation (63), at least in part by inducing rapid T cell disengagement from the DC, effectively raising their activation threshold [reviewed in ref (64)].

Regulatory DC are also well known for their soluble mediators that contribute importantly to their tolerogenic activities. For example, IL-10 is probably the most reported of the inhibitory signals emanating from DCreg (50, 65). It inhibits the antigen-presenting functions of DC (66), but also leads to its own upregulation in these cells. We know that IL-10 expression is essential to the tolerogenicity of IL-10-induced murine DC10 - silencing or deletion of its expression in these cells eliminates their regulatory activities (67, 68). Human and murine DC10 have been reported to suppress Th2 responses *in vitro* and *in vivo* (56, 68–70), perhaps related to IL-10-induced granzyme B expression in these T cells and thereby apoptosis (71). TGFβ secretion also contributes to immune tolerance. Its expression by tumor cells can foster the expression of IDO by endogenous tumor DC and thereby suppress anti-tumor immunity (72), but numerous populations of DCreg have been shown to also express TGFβ and to similarly suppress Teff cells [reviewed in (50)]. Vitamin D/dexamethasone-induced DCreg secretion of TGFβ suppresses both IFNγ production and proliferation of $CD4^+$ T cells from rheumatoid arthritis patients (73), just as TGFβ produced by steady state $CD8\alpha^+$ DC or all-trans retinoic acid-induced DCreg (DC-RA) contributes to suppression of allergic donor Teff cell responses in mouse models (51, 74). TGFβ signaling dampens TCR-induced Ca^{++} influx in T cells, preventing their activation, but it also silences expression of the transcription factors T-bet and STAT4, which are critical to Th1 cell differentiation (72). IL-27 is another cytokine that is intimately linked to the induction of tolerance—it can reduce IL-2 expression during Th1 cell differentiation but also, when coupled with IL-6 signaling, can induce Th1, Th2, and Th17 cells to secrete IL-10 (75). We reported that the DC-RA noted above also secrete high levels of IL-27, but that neutralizing IL-27 does not affect the activation of Th2 cells from peanut allergic mice seen in co-cultures of DC-RA and allergen-presenting immunostimulatory DC. Rather, wild-type (w.t.) DC-RA fully protect against peanut-induced anaphylaxis *in vivo* by driving the differentiation of $LAG3^+CD49b^+Maf^+Foxp3^-$ Treg—unlike w.t. DC-RA, IL-$27^{-/-}$ DC-RA are of no therapeutic benefit (74). IL-27 has also been reported to induce the differentiation of IL-10-secreting Tr1 cells through induction of c-Maf, IL-21 and ICOS in T cells [reviewed in (75)].

DCreg Induce Differentiation of Regulatory T Cells

There are of course multiple populations of $CD4^+$ Treg. $CD25^+Foxp3^+$ Treg, which are probably the most commonly reported Treg, include both naturally-occurring thymic emigrants [natural Treg (nTreg)] as well as cells induced to differentiate in the periphery (induced Treg) from either naïve (76) or effector (77) $CD4^+$ T cells. Interestingly, when the activities of nTreg and DC10-induced Treg of identical TCR specificity were compared in a preclinical model of asthma, the latter cells carried markedly (i.e., ≈5-10-fold) greater regulatory activity than the nTreg (78). There are also inducible IL-10-dependent type-1 Treg (Tr1 cells) that are $CD4^+CD25^-CD49b^+LAG3^+Foxp3^-$ (79), IL-10-independent $CD25^+CD49b^-LAG3^+Foxp3^-$ Treg (74), and oral tolerance-associated, TGFβ-dependent $CD4^+$ Th3 cells (80).

While there is a large body of literature from preclinical models that confirms that DCreg can induce antigen-specific T cell tolerance *in vivo* (50, 65, 81–83), the collective literature addressing the function of human DCreg is less robust. Most studies with human DCreg have been restricted to showing that these DC only poorly activate allogeneic T cell responses *in vitro* (50). Fewer studies have assessed whether human DCreg suppress autologous Teff responses and induce Treg differentiation [e.g., (56)], or critically examined the phenotype and tolerogenic mechanisms of the induced Treg. This is an important issue, for expression of Treg markers in itself does not necessarily mean that the putative Treg are functional. A case in point is a recent report relating to the use of immature human DC-10 to induce tolerance among circulating T cells of peanut-allergic donors. Peanut allergen-presenting DC-10 did indeed induce the differentiation of T cells that expressed the expected Tr1 markers, but these cells lacked Tr1 cell activity, as determined in functional assays (84). Whether this could be related to the recently reported Th2 adjuvant activities of peanut proteins (85) has not been assessed as yet. However, we have shown that allergen-loaded semi-mature DC10 from grass- or cat-allergic donors both suppress Th2 responses and induce the

differentiation of fully functional $CD25^+LAG3^+CTLA4^+Foxp3^+$ Treg (56).

As in many areas of health research, we have gained important insights into the disease processes and therapeutic approaches from lessons learned in animal models. For example, just as IL-10-induced human monocyte-derived DC [whether immature or semi-mature (56, 57)] are tolerogenic *in vitro*, murine bone marrow-derived DC10 are potently tolerogenic *in vitro* and in mouse models of, for example, asthma (68, 69, 77). The regulatory activities of murine DC10 are critically dependent on their expression of IL-10 (67, 68) but also to a lesser extent their expression of CD80 and CD86 (67), IDO (70) and CD40 (Dawicki et al., under review). Interestingly, they induce cognate Th2 cells with which they are cultured to proliferate, but in doing so these Teff cells transdifferentiate into highly effective $CD25^+Foxp3^+$ Treg (77, 78). In contrast, mature retinoic acid-induced DC (DC-RA) induce no proliferative responses among Th2 cells, although the T cells differentiate into potent $LAG3^+CD49b^-Foxp3^-$ Treg (74). Thus, overall it is clear that numerous signals that DCreg can bring to interactions with naïve or effector T cells can induce these cells to take on a regulatory phenotype.

Regulatory T cells are activated in a T cell receptor-dependent fashion but, once activated, they are able to suppress the responses of by-stander T cells through a number of non-specific signals (e.g., secretion of IL-10) (86–91). However, bystander DC can similarly adopt a regulatory phenotype following interactions with Treg, and thereby further foster tolerance through a process known as infectious tolerance (92). Treg have a number of mechanisms by which they can induce DCreg, including CTLA-4 induction of IDO (88), LAG3 activation of inhibitory signals (93), or neuropilin-1 signaling (88). Expression of galectin-1 by $Foxp3^+$ Treg (86) induces DC to secrete IL-10 and IL-27 (94), through which they can foster Tr1 cell differentiation (95). And Treg can themselves recruit additional populations of Treg without a need for a DC intermediary. For example, TGFβ released from Th3 cells triggers development of $CD25^+Foxp3^+$ Treg (96), while LAP-TGFβ on activated $Foxp3^+$ Treg can induce naïve T cells to adopt a similar $Foxp3^+$ Treg phenotype (97).

DCreg Production and Maturation

Currently, the most commonly used approach for generation of human DCreg includes differentiation of purified $CD14^+$ peripheral blood monocytes into immature DC by culture with GM-CSF and IL-4 (98–100). Murine DCreg, thought to belong to the cDC2 category, are similarly differentiated with GM-CSF and IL-4, but most often from bone marrow progenitors (101). In principle, induced pluripotent stem cells could be used to generate very large numbers of DCreg (102–105), but use of such approaches has been tempered by concerns about the potential for pre-existing epigenetic programming in the stem cells and, more on moral grounds, the phenotypic similarities of stem cells to human foetal cells (106–109). All things considered, the use of mo-DCs is presently the industry standard for *in vitro* generation of human DCreg for clinical and experimental applications. Tolerogenic agents [reviewed in (50)] are added to the cultures of differentiating cells at varying times, depending on the type of DCreg in question. Because of the high likelihood that DCreg being used for immunotherapeutic purposes will be exposed to marked inflammatory signals after delivery to the recipient, it is critical that the treatment DCreg not convert into immunostimulatory cells that might exacerbate, rather than ameliorate pathology (110). As such, it is routine that DCreg are assessed for their abilities to withstand such phenotypic conversion following challenge with inflammatory mediators (e.g., IL-1β, IL-6, TNF) or TLR agonists [reviewed in (50)]. Maturational markers for DC include increased expression of HLA-DR, co-stimulatory molecules and inflammatory cytokines (e.g., IL-12) (33, 111–113). On the other hand, inflammation-resistant DCreg retain reduced expression levels of these maturational markers while maintaining their expression of inhibitory receptors and anti-inflammatory cytokine secretion, as noted above (44, 45, 73, 74, 94, 95, 114–117).

There are many well-established protocols to generate DCreg, but it is important to keep in mind that the populations of regulatory cells that we generate include both DCreg and, almost inevitably, additional sub-populations of monocyte-derived progeny that may or may not have their own activities. While some DCreg reports do include, for example, a final $CD11c^+$ DC selection step (e.g., MACS- or FACS-sorting) prior to use of the DCreg generated, the majority of reports do not, suggesting that any effects observed may not be exclusively attributable to the DCreg. For example, a recent report of murine DCreg induction with varying doses of vitamin D (DC-VitD) revealed that there was a dose-dependent output of $CD11c^+$ cells. The control immature DC in these experiments were ≈82% $CD11c^+$, while the DC-VitD comprised up to 92% $CD11c^+$ cells. Thus, while the "DC-VitD" pool of cells strongly suppressed $CD4^+$ T cell activation, potential contributions to that activity of the ≥8% non-DC-VitD were not assessed (63). Depending on the precise culture conditions, culture of $CD14^+$ human monocytes in GM-CSF and IL-4 can lead to differentiation of a mixed population of $CD83^+$ DC, $CD14^+$ macrophage-like cells, and/or myeloid-derived stem cells [reviewed in (118)]. Moreover, while $CD83^+$ DCreg remain the predominant cell in these cultures, a proportion of the $CD14^-$ cells present subsequently regain their expression of CD14 and their macrophage-like properties when exposed to IL-10 and maturational signals. The authors reported that the presence of even small numbers of these contaminating $CD14^+$ macrophage-like cells skewed the apparent regulatory phenotype of the "DCreg population" in a dose-dependent fashion (118). It has also been reported that human DC10 generated from plastic-adherent monocytes comprise two major sub-populations, including $CD83^{hi}HLA\text{-}DR^{hi}CCR7^+$ cells that strongly express CD25, and $CD83^{lo}HLA\text{-}DR^{lo}CCR7^{lo}$ cells. Both populations can suppress Teff cell proliferation and induce Treg differentiation, but the $CD83^{hi}HLA\text{-}DR^{hi}$ population is significantly better in both tasks, at least in part because the regulatory activities in these DC10 cultures were CD25-dependent (119). Taken together, these outcomes do not diminish the importance of DCreg, but

they do raise the question of whether contaminating cells in, for example, monocyte-derived cultures might also contribute significantly, but in a negative manner, to the regulatory outcomes observed. This would be relevant for functional studies, but it would be critical to have such insights when undertaking, for example, global transcriptomic studies of DCreg.

DCreg Control of Tolerance Responses

In order for DCreg to be clinically relevant, the protocols used for their induction must be optimized, keeping in mind the intended target disease. This is in part because the types of infectious tolerance processes launched by the chosen DCreg must fit the clinical indication. For example, as suggested above, both DC-VitD/dex and DC10 secrete IL-10 and thereby induce Th2 cells to differentiate into $CD25^{+}Foxp3^{+}$ Treg which can fully reverse the asthma phenotype (68, 69, 77, 120). On the other hand, as noted above DC-RA production of IL-10, TGFβ, and IL-27 suppresses peanut anaphylaxis-inducing Th2 cells through induction of $LAG3^{+}CD49b^{-}Foxp3^{-}$ Treg (74). The relevance of this distinction lies in the observation that intestinal inflammation can suppress Foxp3 expression in a mouse model of colitis, such that intestinal $Foxp3^{+}$ Treg therein convert into pathogenic Th17 cells (121). This begs the question of whether use of DCreg strategies that induce $Foxp3^{-}$ Treg, which could be more inflammation-resistant than the $Foxp3^{+}$ Treg, would be better suited in this context. Similar inflammation-associated adverse outcomes have been reported in other DCreg immunotherapy models (122, 123). Having said that, it has also been reported that CD40/CD80/CD86-knock down DCreg also induce the differentiation of $Foxp3^{+}$ Treg in a murine colitis model, but that this treatment is successful in preventing leukocyte infiltration and disease development (124). This indicates that, while it is critical that they be taken into consideration, inflammatory conditions that may be seen by treatment DCreg *in vivo* do not necessarily lead to adverse outcomes, and that bodes well for DCreg immunotherapeutics.

DCreg routinely recruit Treg into infectious tolerance processes, but induction of Treg is not an essential facet of DCreg-induced tolerance. For example, it has been reported that human vitamin D-induced DCreg foster allogeneic T cell differentiation into classical Treg (125–128), but also that at least some forms of vitamin D-induced DCreg can instead foster a Treg-independent type of tolerance. Thus, murine $CD11c^{+}$ DC that had been differentiated in GM-CSF and vitamin D, or human DCreg differentiated from $CD34^{+}$ stem cell precursors with vitamin D do not induce Treg responses, but instead impair $CD4^{+}$ T cell priming in a CD31-dependent fashion (63). Silencing of CD31 increases T cell activation in DCreg: T cell co-cultures, while its overexpression leads to substantially reduced DC:T cell contact times, with reductions in IL-2 production by the T cells and a consequent loss of T cell priming (63). Collectively this evidence suggests that we must be cognizant that even seemingly closely-related populations of DCreg may utilize quite disparate regulatory mechanisms, and that should be taken into consideration when planning immunotherapeutic approaches. Although such variance may be difficult to predict, there are emerging trends in the published literature.

Tables 1 and **2** outline our more recent advances in the biology of non-human (murine, NHP) and human DCreg, respectively. Because we comprehensively reviewed this area previously (50), these tables provide only an update on observations regarding novel agents to induce the DCreg, or the use of previously reported DCreg in new models, rather than a comprehensive listing of all types of DCreg investigated to date. Thus, the interested reader can find additional information on human DCreg found across our organ systems and those induced *in vitro* with IL-10 (6, 56, 57, 68–70, 153–161) or other cytokines (160–165), corticosteroids (156, 166–168), vitamin D3 (127, 167, 169–172), rapamycin (156, 167, 173, 174), and neuropeptides (175, 176) in the references cited. **Table 1** provides *in vivo* data from animal models of human diseases, and includes the agents used to induce and mature murine and NHP DCreg, the disease model addressed, the phenotype of the DCreg and the mechanisms by which it induces tolerance, its clinical effects in that model and whether it activates secondary regulatory processes (e.g., Treg).

Table 2 provides data from human DCreg generated from $CD14^{+}$ monocytes *in vitro*, and includes the agents used to induce and mature the cells, the phenotype of the DCreg, the mechanisms by which they induce their immunologic effects and whether they activate Treg. While the regulatory activities for most of these cells were assessed exclusively *in vitro*, the IL-10-lentivirus-tranfected human DC10 were also assessed for their abilities to protect humanized otherwise immunocompromised mice from graft-versus-host disease (149).

Impact of Microbial Exposures on the Phenotype of DCreg

There are a number of questions that should be addressed before DCreg immunotherapy can become mainstream as a clinical approach. As suggested above, a particularly important one is the nature of inflammatory conditions the DCreg may face after delivery to the host and their impact on the cell's regulatory activities. We have previously reviewed the array of innate immune receptors expressed by DC, which include protease-activated receptors (PARs), TLR, C-type lectin receptors, retinoic acid-inducible gene-1 (RIG-1), and the melanoma differentiation-associated gene-5 (MDA-5) (50), but they also express receptors for an array of other inflammatory mediators (177, 178). As noted previously, there are multiple reports that the tolerogenic potential of DCreg can be arrested, or even reversed, under inflammatory conditions, such that the cells instead activate effector T cells and thereby exacerbate pathology (122, 123, 179). Despite the fact that many microbial stimuli have adverse effects on DCreg, there is an accumulating body of evidence that other microbial signals contribute importantly to the regulatory activities of these cells. For example, delivery of the TLR5 agonist flagellin to asthmatic mice ameliorates their disease phenotype through induction of

TABLE 1 | Studies examining induced non-human regulatory DC.

Induction Agent (Mat. Agent)	Species (Admin. Route)	Disease Model	DC Phenotype	Mechanism of Tolerance	Performance Outcomes	Secondary Induction of Tolerance	Reference
Murine models							
9-cis-retinoic acid (LPS)	Mouse (s.c.)	Contact hyper-sensitivity	↓ CD103, CD207, MHCII, CD80, CD86 ↑ PD-L1, osteopontin	Osteopontin-dependent	↓ contact hyper-sensitivity	↑ $CD25^+$ $Foxp3^+$ Treg	(129)
all-trans-retinoic acid (LPS)	Mouse (i.p.)	Food (peanut, egg) allergies	↑ MHCII, TGFβ, Aldh1A2, PD-L2, IL-27	Th2 anergy, IL-10 & TGFβ-dependent; Treg induction, IL-27-dependent	↓ anaphylactic responses	↑ $CD25^+$ $LAG3^+CD49b^-$ $Foxp3^-$ Treg	(74)
Allograft inhibitory factor-silenced DC (LPS)	Mouse (s.c.)	OTII TCR-transgenic T cell responses *in vivo*	No discernible changes in DC marker or cytokine secretion vs. w.t. DC-LPS	–	↓ T cell proliferation & IL-2, IFNγ & TNF secretion	↑ $CD4^+CD25^+$ $Foxp3^+$ Treg	(130)
c-reactive protein (LPS)	Mouse (N/A)	EAE	↓ MHCII/CD86/CD40 ↓ CD11b+CD11c+BMDC ↓ IL-10/IL-12p70 production	FcγRIIB-dependent	↓ Antigen-specific T cell proliferation	–	(131)
CD40 siRNA-silenced	Mouse (i.p., i.n., i.v., or s.c.)	Allergic Rhinitus	↓ IL-4/IL-5 production ↑ IL-10, IL-35 production	N.D.	↓ symptoms, & tissue IL-4, IL-5 & eosinophils (i.n. DCreg only)	↑ IL-10, IL-35 & FoxP3 expression	(132)
CD40/80/86-silenced DC (N/A)	Mouse (i.p.)	Colitis	–	–	Prevention of colitis development ↓ Leukocyte infiltration to colon	↑ $FoxP3^+$ Treg ↑ $IL\text{-}10^+$ Breg	(124)
Dexamethasone (LPS)	Mouse (i.p.)	chronic Chagas disease cardio-myopathy	↓ MHCII/CD86/CD80/CD40 ↓ IL-6/IL-12 ↑ IL-10 production	–	↓ Heart inflammation & fibrosis ↓ IL-2/IFNγ *in vitro*	↑ $CD4^+FoxP3^+$ Treg	(133)
Dexamethasone (LPS)	Mouse (i.p.)	Dust Mite Allergy	↓ MHCII/CD86/CD80/PD-L2 ↓ IL-6/IL-12 ↑ IL-10 production	–	↓ Inflammatory cells in lungs and mucus deposition ↓ Antigen-specific IgE ↓ *Gata3* & *IL-4* in lungs	↑ $FoxP3^+$ Treg	(134)
Dexamethasone (IFN-γ & LPS)	Rat (N/A)	–	↓ MHCII/CD86/CD80/IL-6/IL-12 ↓ Inducible NO synthase ↑ Sialic acid upregulation	Partially sialic acid-dependant	↓ allogeneic T cell proliferation & activation	–	(135)
Minocycline (IFN-γ & TNF-α or LPS)	Mouse (i.v.)	EAE	↓ MHCII/CD54/CD80/CD86/ IL-1β/IL-6/IL-12/TNF ↑ PD-L1	–	↓ Allogeneic T cell priming ↓ EAE clinical responses	↑ $CD4^+$ $CD25^+FoxP3^+$	(136)
Minocycline & Dexamethasone (IFN-γ & TNF-α or LPS)	Mouse (i.v.)	EAE	↓ MHCII/CD54/CD80/CD86/ IL-1β/IL-6/IL-12/TNF	Partially IL-10-dependant	↓ Antigen-specific T cell proliferation ↓ Clinical responses	↑ $FoxP3^+$ Treg	(137)
IL-10	Mouse (i.p., s.c., i.v., t.t.)	Asthma	↓ MHCII/CD80/CD86/IL-12 ↑ IL-10	↓ Th2 response. ↓ B cell response. - Convert Teff to $CD25^+Foxp3^+$ Treg - IL-10-dependent	i.p. or t.t. eliminate asthma phenotype s.c. ↓ Th2 response	↑ $FoxP3^+$ Treg	(68–70, 77)

(Continued)

TABLE 1 | Continued

Induction Agent (Mat. Agent)	Species (Admin. Route)	Disease Model	DC Phenotype	Mechanism of Tolerance	Performance Outcomes	Secondary Induction of Tolerance	Reference
Lentiviral- IL-10 upregulation (LPS)	Mouse (i.v.)	GVHD	↑ IL-10 production	–	↓ *In vitro* T cell proliferation & activation ↑ Survival times ↓ GVHD scores	↑ $CD4^+CD25^+$ $LAG3^+CD49b^+FoxP3^-$ Tr1 cells ↑ IL-10, IFN-γ	(138)
RelB-silencing (LPS or CpG-B DNA & CD40L)	Mouse (N/A)	SLE	↓ MHCII/CD40/CD86 ↓ IL-12/TNF	–	↓ Ag-specific splenic T cell activation ↑ IL-10 production ↓ IFN-γ/IL-17 production	Possible $FoxP3^-$ IL-10-producing Treg	(139)
METTL3-silencing (LPS)	Mouse (i.v.)	Heart transplant	↓ MHCII/CD80/CD86 ↓ IL-12/IFN-γ ↑ IL-4/10 production	–	↓ Th1:Th2 ratio ↓ Survival times ↓ pathological scores	–	(140)
Vitamin D & Dexamethasone (LPS)	Mouse	proteoglycan-induced Arthritis	↓ MHCII ↓ PD-L1/CD86 ratio ↓ IL-10 production	–	↓ Antigen-specific T cell proliferation ↓ Clinical responses	↑ $FoxP3^+$ Treg	(141)
NON-HUMAN PRIMATE							
***VitD3* & IL-10 (TNF/IL-1/IL-6)**	Rhesus macaque (i.v.)	Renal transplant	↑ HLA-DR/CD86 ↑ PD-L1	↓ Allo-T cell proliferation ↓ IL-17 production	↑ graft survival times (NS)	↑ $CD4^+$ $CD25^+FoxP3^+$ $CTLA4^{hi}$ Treg	(141–146)

Mat. Agent, maturation agent; Admin. route, administration route; LPS, lipopolysaccharide; EAE, experimental autoimmune encephalomyelitis; BMDC, bone marrow-derived DC; Breg, regulatory B cell; NO, nitric oxide; GVHD, graft-versus-host disease; SLE, systemic lupus erythematosus; METTL3, methyltransferase-like protein 3; VitD3, 1,25-dihydroxyvitamin D3.

TABLE 2 | Studies examining induced human regulatory DC.

Induction Agent (Mat. Agent)	DC Phenotype	Mechanism of Tolerance	Regulatory effects	Secondary Induction of Tolerance	Ref
Dexamethasone/*VitD3* (LPS)	↓ CD83/CD86/CD40	↓ Akt and p38MAPK protein phosphorylation	↓ Autologous T cell proliferation ↓ Th1/Th17 Differentiation	↑ $FoxP3^+$ IL-10-secreting Treg	(147)
Dexamethasone/*VitD3* (LPS)	↓ CD80/CD83/CD86 ↑TGF-β1 production	Partially TGF-β1-dependant	↓ Allogeneic $CD4^+$ T cell proliferation ↓ IFN-γ production	–	(73)
Ethyl pyruvate (TNF, IL-1, PGE2)	↓ CD40/CD83/CD86/HLA-DR ↓ IL-6, IL-12, TNF-α production	–	↓ Allogeneic $CD4^+$ T cell proliferation ↓ IL-17/IFN-γ production	–	(148)
IL-10 (TNF, IL-6, IL1, PGE2)	Two DC sub-populations: **i -** $CD83^{hi}CCR7^+HLA\text{-}DR^{hi}$ ↑ CD25 (membrane & soluble) **ii -** $CD83^{lo}CCR7^-HLA\text{-}DR^{lo}$ Lower CD25	Largely CD25-dependant	↓ Allogeneic $CD4^+$ T cell proliferation with both $CD83^{hi}$ $HLA\text{-}DR^{hi}$ > $CD83^{lo}HLA\text{-}DR^{lo}$ cells	$CD83^{hi}$ $HLA\text{-}DR^{hi}$-induced Treg more potent than $CD83^{lo}HLA\text{-}DR^{lo}$-induced Treg	(119)
IL-10-lentivirus (TLR agonists or TNF-α, IL1β and IL-6)	↑ CD14/CD16/CD141/CD163/HLA-DR ↑ ILT4/HLA-G ↓ CD1a ↑ IL-10 production	–	↓ Allogeneic $CD4^+$ & $CD8^+$ T cell proliferation ——— NSG mouse model: ↓ Severity of GVHD ↑ GVHD survival time	↑ Treg (Tr1)	(149)
Retinoic acid (LPS)	↓ CD80/CD86/HLA-DR ↑ CD141, GARP	CD141 & GARP-dependent	↑ Allogeneic $CD4^+$ T cell IL-10	↑ $CD4^+CD25^+$ $FoxP3^+$ Treg	(150)
Staph peptides (LPS or staph cell lysate)	↓ CD40/CD80/HLA-DR ↓ IL-10, IL-12, TNF-α	Possibly ↓ NF-κB and p38 pathway phosphorylation	↓ Antigen uptake by DC ↓ autologous Th1 priming ↓ IFN-γ production ↑ IL-13 production	↑ $CD4^+$ $CD127^-$ $CD25^{hi}$ $CD45RA^-$ $FoxP3^{hi}$ iTreg	(151)
***VitD3* (IFN-γ & LPS)**	↓ HLA-DR/CD86/CD80 ↑ PD-L1/ILT-3/CD52 ↓ IL-10/TNFα/IL-12 production	–	↓ allogeneic T cell proliferation *in vitro* ——— NSG mouse model: ↓ Severity of GVHD with autologous PBMC reconstitution	↑ $FoxP3^+$ Treg	(125)
***VitD3* (+/- manipulation of CD31) (MBP +/- LPS)**	-	↑ CD31 expression	↓ Autologous $CD4^+$ T cell priming ↓ $CD4^+$ T cell interaction time ↓ IL-2/IFN-γ/GM-CSF/TNFα production	–	(152)
***VitD3* & IL-10 (LPS, CD40L or TNF/IL-6/IL-1β/PGE2)**	↓ CD80/CD86 ↑ PD-L1 ↑ IL-10 & IL-12 production	Partially PD-L1-dependant	↓ Allogeneic $CD4^+$/$CD8^+$ T cell proliferation ↓ IFN-γ/IL-17a/perforin /granzyme B production	–	(62)

Mat. Agent, maturation agent; VitD3, 1,25-dihydroxyvitamin D3; LPS, lipopolysaccharide; GVHD, graft-versus-host disease; PGE2, prostaglandin E2.

DCreg and, subsequently, Treg responses (180). Moreover, exposure of monocyte-derived DC from house dust mite (HDM)-asthmatic individuals to flagellin also empowers the DC to take on a regulatory phenotype. These flagellin-induced DCreg express high levels of IL-10, TGFβ and HLA-G, such that they strongly foster $CD25^+Foxp3^+$ Treg responses (180). In a similar manner, delivery of the dectin-1/TLR2 agonist curdlan (181), a bacterial cell wall exopolysaccharide, to asthmatic mice induces differentiation of IL-10-expressing, Maf^+Foxp3^- Treg (i.e., Tr1 cells), and thereby reverses the animal's asthma phenotype (182). On the other hand, human IL-10-lentivirus-transfected DCreg display very complex responses to microbial agonists (149), including polyinosinic:polycytidylic acid (Poly I:C), LPS, flagellin and CpG (agonists specific for TLR3, TLR4, TLR5, and TLR9, respectively), as well as *Listeria monocytogenes* (149). The authors of this report found that a number of classical DCreg markers were differentially regulated on exposure to these agonists. For example, poly I:C challenge increased HLA-G and decreased ILT4 expression, while LPS exposure down-regulated HLA-G and up-regulated ILT4. In contrast to IL-10-lentivirus-transfected murine DCreg, which induce differentiation of $CD25^+Foxp3^+$ Treg (159), the IL-10-lentivirus-transfected human DCreg instead induced the development of Tr1 responses (149). Interestingly, however, while the numbers of Tr1 marker-positive cells were differentially affected by the microbial agonists added to the human DCreg-T cell cultures, marked differences in the suppressive activities or cytokine profiles of the Tr1 cells so induced were not observed (149).

The recognition that our microbiome contributes importantly to our overall health has brought its impact on immune regulation by DC into focus. We know, for example, that the diversity of the microbiome in neonates of ≤100 days of age can predict their likelihood of developing asthma as children (183). Reduced levels of *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* genera bacteria in the neonatal gut are associated with an increased risk of asthma. The neonates so affected display reduced levels of fecal acetate, but see increased urinary excretion of a number of secondary bile acids with potential links to this intestinal dysbiosis (183). We know that microbial catabolites (e.g., short-chain fatty acids) such as butyrate and propionate can induce a tolerogenic phenotype in DC, dampening LPS-induced expression of costimulatory molecules (e.g., CD83, CD40) and augmenting downstream Tr1 responses (184). And culture with *Lactobacillus paracasei* L9 induces murine bone marrow-derived DC to strongly upregulate IL-10 secretion and to take on a regulatory phenotype, such that they induce $CD4^+$ effector T cells from β-lactoglobulin-allergic mice to differentiate into $Foxp3^+$ Treg (185). Similarly, in experimental germ-free mice β-glucan/galactan polysaccharides and polysaccharide A secreted by intestinal *Bifidobacterium bifidum* strain PRI1 are associated with increased expression of IL-10, TGFβ, IDO, and PD-L2 within the lamina propria $CD103^+$ DC population (186). Importantly, these DC in turn induce the development of Treg with TCR specificity not only for *B. bifidum*, but also for otherwise unrelated intestinal antigens (186). And *Bifidobacterium infantis* colonization has been reported to induce shrimp tropomyosin tolerance in a mouse model of shrimp allergy, increasing the gut population of $CD103^+$ DCreg, expression of IL-10 and TGFβ, and the numbers of $CD4^+CD25^+CD127^-$ Treg (187).

Unlike pathogenic enteric bacteria, which actively secrete pathogenic mediators within the gut, tolerance-promoting gut symbionts reportedly interact with their hosts *via* capsular polysaccharides that decorate outer membrane vesicles (OMV) shed by the bacteria. Thus, *Bacteroides fragilis* OMV signal to gut DC through TLR2, which induces the development of DCreg and Treg (188), but there can be heterogeneity in the way that gut DC respond to such signaling. Thus, while *Bacteroides thetaiotaomicron* OMVs induce IL-10 expression and a regulatory phenotype in colonic DC of healthy subjects, such immune regulation does not occur with DC from individuals with ulcerative colitis (189). Organisms with beneficial properties can also be found in the external environment. Indeed, curdlan, the tolerogenic β-glucan noted above (182), is derived from the environmental bacterium *Alcaligenes faecalis*. In a similar manner, exopolysaccharides from *Cyanobacterium aponinum*, an organism represented at high levels in the waters of the Blue Lagoon of Iceland, induce human DC to express IL-10 and CD141, leading to Treg induction (190, 191). Bathing in the Blue Lagoon is renowned for its abilities to alleviate psoriatic plaques (191).

Regulatory DC for Clinical Trials

There have been scores of preclinical studies in mouse models documenting that DCreg can induce immunologic tolerance and reverse the disease phenotype in models of allergy and asthma, autoimmune diseases and transplant rejection responses [reviewed in (50)]. As part of moving DCreg closer to clinical application there have also been a number of DCreg trials undertaken in NHP models (**Table 1**). An early study with maturation-resistant VitD3/IL-10-induced rhesus macaque DCreg showed that cells given i.v. led to an initial increase in recipient blood levels of anti-donor and -third party T cell reactivity, but this subsequently waned to below pre-treatment levels in animals also treated with the clinical immunosuppresant CTLA4Ig. There was no induction of anti-donor IgG or IgM alloantibodies detected and, in animals given both DCreg and CTLA4Ig, alloreactive IL-10-producing T cells were detected at ≥28 days post-infusion (143). Culture of such VitD/IL-10-induced NHP DCreg with purified allogeneic peripheral blood $CD4^+CD127^{lo}$ cells, which were enriched in Foxp3-expressing cells (i.e., Treg), for up to 2 weeks led to an expansion of the $CD4^+CD127^{lo}$ Treg, and these in turn induced a dose-dependent suppression of $CD4^+$ Teff cell responses (144). A subsequent study with kidney-engrafted rhesus macaque monkeys treated with donor-derived VitD3/IL-10 DCreg showed that the DCreg significantly prolonged graft survival (from ≈39 to 113 dy) and reduced donor-reactive memory T cell:regulatory T cell ratios, with no evidence of circulating donor-specific alloantibodies (146). In another study, VitD/IL-10-induced kidney transplant recipient DCreg were pulsed with donor-derived PBMC membrane vesicles and used to treat

transplanted monkeys that were also administered CTLA4Ig and rapamycin. Overall, the authors observed ≈2-fold-prolonged graft rejection times, attenuated systemic IL-17 levels, and modulated $CD4^+$ and $CD8^+$ T cell responses to donor antigens, although these were not statistically significant effects (142). The most recent NHP study reported addressed the impact of VitD/IL-10 DCreg treatment on $CD4^+CTLA4^{hi}$ (i.e., Treg) in CTLA4Ig-treated renal allo-transplant recipients. They found that in the absence of DCreg therapy, CTLA4Ig treatment led to reductions in the numbers of circulating $CD4^+CTLA4^{hi}$ Treg, while these reductions were not observed in DCreg-treated recipients (145). Overall, these studies highlighted the potential utility of DCreg immunotherapy in preventing rejection of organ transplants [reviewed in (192)].

Consistent with the outcomes of these NHP DCreg studies, there have been a number of successful Phase I or I/II human DCreg clinical trials completed by pioneers in this area in recent years (**Table 3**). There are reportedly additional trials that are still in recruitment stages or that have not yet published their results (200), as well as several long-term trials ongoing in organ transplant recipients [reviewed in ref (192)]. As these have been largely Phase 1 or I/II trials, their aims were solely to determine whether the DCreg immunotherapy approach in question is safe and well-tolerated, and it is clear that that is the case—the vast majority of subjects have not suffered significant adverse events (193, 195–199). Although, as might be expected for such trials, abrogation of disease was not seen under any of these protocols, reductions in symptoms scores were reported in a small subset of treatment group subjects in the rheumatoid arthritis (195, 197) and Crohn's disease (196) trials. The observed changes in immunologic parameters in the treatment groups across these studies were encouraging, given that these were Phase I studies, though quite modest (**Table 3**).

Human monocyte-derived DC are, overall, $HLA\text{-}DR^+$ $CD11c^+BDCA3^-$ and express an array of other prototypical DC markers (28) but there is substantial heterogeneity in the markers expressed by, and activities associated with any one DC population. Even seemingly very small differences in the DC culture conditions, from how the starting $CD14^+$ monocyte population is isolated and its purity, to the concentrations of GM-CSF or IL-4 employed, the type of serum or serum-free supplement used in the cultures, times in culture, etc., can have very significant effects on the DC generated. This raises the academic question of whether differentiated tissue DC (e.g., blood, tonsillar) might be better candidate cells for DCreg immunotherapeutics—arguably their use could call for fewer investigator manipulations of the cells. Nevertheless, global transcript profiling of monocyte-derived DC, a number of DC cell lines and purified tissue DC reveals that there is substantial phenotypic heterogeneity here too. Monocyte-derived cells cluster in principle component analyses closely with tonsillar DC, but not other populations, while the blood DC populations tend to loosely cluster by themselves (201). However, developing a DCreg therapy using primary populations of DC would bring its own challenges, not the least of which is the relative scarcity of the cells. Fully differentiated blood DC constitute only 0.1%–1.0% of peripheral blood mononuclear cells (9, 202), such that it would not likely be feasible to purify sufficient numbers of primary cells from any one donor for an effective treatment. Thus, at this point in time, use of well-characterized and standardized monocyte-derived populations of DCreg would appear to be our best clinical option.

One of the more important factors in optimizing DCreg immunotherapy is identification of DCreg of a phenotype that best fits the intended application. There have been a number of reports that have run head-to-head comparisons of human DCreg differentiated with an array of mediators, including comparisons of vitamin D3-, dexamethasone- and rapamycin-induced cells (167), or of vitamin D3-, IL-10-, dexamethasone-, TGFβ– and rapamycin-induced DCreg (156), and assessments of DCreg differentiated with protein kinase C inhibitor versus dexamethasone, vitamin D3, rapamycin, IL-10, TGFβ or a combination of peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist and retinoic acid (203). This has provided important insights into DC yields & viabilities, expression of co-stimulatory or inhibitory markers, IL-10 production, resistance to inflammatory stimuli, and abilities to activate allogeneic T cells or induce Treg (156, 167). Nevertheless, multiple questions remain even given our knowledge and advances in DCreg immunotherapy. While it is easy to appreciate the simplicity of using allogeneic cells for functional readouts with human DCreg, we question whether assessing their impact on autologous, disease-related T cells would more directly address the question of the cell's suitability for immunotherapeutic applications (i.e., other than in the context of allogeneic transplantation). That is, given that the *raison d'etre* for DCreg therapy is more often than not suppression of specific antigen-driven pathology, would assessing the cell's abilities to suppress autologous cognate T cell responses be more relevant (197)? Is assessing expression of Treg-associated markers (e.g., Foxp3) adequate to conclude that the putative Treg induced by these DCreg treatments are functional as regulatory cells? As noted above, at least some DCreg can induce differentiation of T cells that express Treg markers as determined by FACS, despite the fact that they are not functional regulatory cells (84). And, given that different DCreg use different strategies to induce tolerance (204) and thereby induce distinct types of Treg (50), should the nature of the condition being treated (e.g., low versus high levels of target tissue inflammation) impact the choice of the DCreg to be employed? As alluded to above, are there indications that we should target selective induction of $Foxp3^+$ versus $Foxp3^-$ Treg or, for that matter, $CD4^+$ versus $CD8^+$ or other regulatory cells? Another question is whether *in vitro* assays of DCreg function provide sufficiently robust data on the activities of these cells to validate them as candidates for clinical trials. Could *in vivo* modeling in, for example, humanized mice (205) or NHP (142–146) provide more meaningful insights?

While we often assess a standard set of markers associated with DCreg, it is clear that functional DCreg are more than cells that express low levels of CD40 or CD86, or high levels of PD-1 and IL-10. Indeed, numerous transcriptomic analyses have

TABLE 3 | Human DCreg clinical trials.

Induction Agent (Mat. agent)	Status (n)	Disease target	DC-loading antigen	Method of DC Administration (Final Dose)	Performance Outcomes	Clinical Outcomes	Reference
i. CD40/CD80/CD86-silenced ii. control DC (none)	Phase 1; Completed (n = 10)	Type 1 diabetes	Autologous -	Intradermal (4×10^7)	↑ $B220^+CD11c^-$ B cells in both control and exp groups	No adverse events, no clinical changes	(193)
Semi-mature DC with rheumatoid antigens	Phase 1; Completed (n = 12)	Rheumatoid arhtritis	PAD4, RA33, citrullinated-filaggrin, vimentin	Subcutaneous (5 doses each: low dose, 5×10^6; high dose, 1.5×10^6)	↓ IFN^+ T cells & autoantibodies EULAR response, ↓ 33% to 83% (low vs. high dose)	C dose-dep effects (58% of subj had reduced RA scores)	(194) abstract only
NF-κB inhibitor (N/A)	Phase 1; Completed (n = 34; 16 control)	Rheumatoid arthritis	Citrullinated Peptides (4)	Intradermally (7.2×10^3 – 6.2×10^4)	↑ Treg:Teff cell ratios ↓ Serum IL-15, IL-29, CX3CL1 and CXCL11 ↓ T cell IL-6	Reduction of disease symptoms within 1 month of treatment	(195)
Dex/Vit A (IL-1β, IL-6, TNF-α, & PGE2)	Phase 1; Completed (n = 9)	Crohn's Disease	–	Intraperitoneal ($2/5/10 \times 10^6$ or $6/15/30 \times 10^6$)	↓ IFNγ (FACS) ↑ blood Th17 & $Foxp3^+$ T cells	Reduction of disease severity within 12 weeks of treatment	(196) -
Dex/VitD3 (MPLA)	Phase 1; Completed (n = 13)	Rheumatoid arthritis	Autologous synovial fluid	Intra-articular ($1/3/10 \times 10^6$)	–	Reduction of disease symptoms for 2 patients	(197)
Dex (IL-1β, IL-6, TNF-α, & PGE2)	Phase 1b; Completed (n = 8)	Multiple Sclerosis	Myelin Peptides (7)	Intravenous ($50/100/150/300 \times 10^6$)	↓ $CD8^+$ & NK cells ↑ PBMC IL-10	–	(198)
Dex (IL-1β, IL-6, TNF-α, & PGE2)	Phase 1b; Completed (n = 4)	NMOSDs	Myelin Peptides (7) + AQP4	Intravenous ($50/100/150/300 \times 10^6$)	↑ PBMC IL-10 ↓ *In vitro* antigen-specific T cell proliferation	–	(198)
Dex/IL-10	Phase I (n = 15)	Liver transplant	Donor-autologous	Intravenous ($2.5–10 \times 10^6$/kg)	↓ $T\text{-}bet^+Eomes^+CD8^+$ T cells ↑ $Foxp3^+$ Treg: $T\text{-}bet^+Eomes^+$ CD8 T cell ratios (pre-transplant)	No adverse reactions to DCreg infusion	(199)

AQP4, aquaporin-4; Dex, dexamethasone; EULAR, European League Against Rheumatism; Mat. agent, maturation agent; MPLA, monophosphoryl lipid A; NMOSDs, neuromyelitis optica spectrum disorders; PGE2, prostaglandin E2; Vit A, vitamin A; VitD3, 1,25-dihydroxyvitamin D3.

identified many hundreds of markers that are differentially expressed by DCreg (170, 206–210) but only three immunologically-relevant genes [annexin A1, glucocorticoid-induced leucine zipper (GILZ) and IDO] have been reported to display differential increases in expression within the DCreg across five or more of these reports, while several more (cathepsins B, C, D & L, ILT3, stabilin 1, and TGFβ) saw increases across three of these reports (211). Nevertheless, it is interesting that IL-10 expression, as an example, has not been so identified, particularly given its intimate association with DCreg across a spectrum of reports [reviewed in (50, 65)]. Are there other leads we are missing in this search? Examination of the blood DC from allergic individuals who have undergone allergen-specific immunotherapy have identified a number of markers associated with successful tolerance induction (e.g., C1q, stabilin-1) (210). Indeed, administration of C1q in mouse models of asthma was subsequently shown to also effectively reduce the asthma phenotype in these animals (212). Intuitively, it seems that each population of DCreg likely enjoys contributions from a large number of regulation-associated inputs (e.g., expression of inhibitory mediators and receptors, low levels of HLA-DR and co-stimulation, etc.) that cumulatively define the cell's regulatory activities, with different populations of DCreg employing distinct levels of large numbers of these as unique inputs.

CONCLUSION

Currently, the standard of care for allergies and autoimmune disorders comprises the use of immunosuppressive drugs, agents which often have substantial deleterious side-effects and must be administered for the life of the patient. Regulatory dendritic cell research is promising in that DCreg-induced immune regulation appears to be robust and enduring (70), at least as far as we have been able to ascertain in animal models. Murine DCreg treatments can reverse or eliminate even severe disease in models of allergies and autoimmune diseases and extend the life of organ transplants, sometimes indefinitely. They do so by activating infectious tolerance processes that incorporate secondary induction of different types of Treg which, in turn, can recruit additional generations of regulatory cells. Though animal model outcomes cannot be directly translated to human conditions, the burgeoning *in vitro* research and early-stage clinical trial outcomes with human DCreg bode well for their use in the clinic. Certainly, our DCreg clinical trials to date indicate that DCreg immunotherapy is safe and well-tolerated. Our increasing advances in the cellular and molecular biology of these cells is likely to have a significant impact on the efficacy of DCreg in the clinic in the foreseeable future. Unlike our current pharmacological management of these diseases, immunotherapy with DCreg would seem to allow for the possibility of long-term restoration of the physiologic equilibrium associated with immunologic tolerance.

AUTHOR CONTRIBUTIONS

SN wrote the first drafts of the manuscript. SL contributed to the literature searches. JG contributed to the writing and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Funding was provided by grants from the Canadian Institutes of Health Research (MOP53167) and the AllerGen Networks of Centers of Excellence (16B&B10).

REFERENCES

1. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* (1973) 137(5):1142–62. doi: 10.1084/jem.137.5.1142
2. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J Exp Med* (1974) 139(2):380–97. doi: 10.1084/jem.139.2.380
3. Steinman RM, Kaplan G, Witmer MD, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J Exp Med* (1979) 149(1):1–16. doi: 10.1084/jem.149.1.1
4. Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* (1978) 75(10):5132–6. doi: 10.1073/pnas.75.10.5132
5. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* (1991) 9:271–96. doi: 10.1146/annurev.iy.09.040191.001415
6. Enk AH, Angeloni VL, Udey MC, Katz SI. Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. *J Immunol* (1993) 151(5):2390–8.
7. Simon JC, Hara H, Denfeld RW, Martin S. UVB-irradiated dendritic cells induce nonproliferating, regulatory type T cells. *Skin Pharmacol Appl Skin Physiol* (2002) 15(5):330–4. doi: 10.1159/000064537
8. Crowley M, Inaba K, Witmer-Pack M, Steinman RM. The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell Immunol* (1989) 118(1):108–25. doi: 10.1016/0008-8749(89)90361-4
9. Freudenthal PS, Steinman RM. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc Natl Acad Sci U S A* (1990) 87(19):7698–702. doi: 10.1073/pnas.87.19.7698
10. Heidkamp GF, Sander J, Lehmann CHK, Heger L, Eissing N, Baranska A, et al. Human lymphoid organ dendritic cell identity is predominantly dictated by ontogeny, not tissue microenvironment. *Sci Immunol* (2016) 1 (6):eaaai7677. doi: 10.1126/sciimmunol.aai7677
11. Granot T, Senda T, Carpenter DJ, Matsuoka N, Weiner J, Gordon CL, et al. Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics over Human Life. *Immunity* (2017) 46(3):504–15. doi: 10.1016/j.immuni.2017.02.019
12. Guilliams M, Dutertre CA, Scott CL, McGovern N, Sichien D, Chakarov S, et al. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity* (2016) 45(3):669–84. doi: 10.1016/j.immuni.2016.08.015
13. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol* (2014) 14(8):571–8. doi: 10.1038/nri3712
14. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells

in human peripheral blood. *J Immunol* (2000) 165(11):6037–46. doi: 10.4049/jimmunol.165.11.6037
15. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* (2005) 23:275–306. doi: 10.1146/annurev.immunol.23.021704.115633
16. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN. Characterization of human blood dendritic cell subsets. *Blood* (2002) 100 (13):4512–20. doi: 10.1182/blood-2001-11-0097
17. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood* (2010) 116 (16):e74–80. doi: 10.1182/blood-2010-02-258558
18. Auray G, Keller I, Python S, Gerber M, Bruggmann R, Ruggli N, et al. Characterization and Transcriptomic Analysis of Porcine Blood Conventional and Plasmacytoid Dendritic Cells Reveals Striking Species-Specific Differences. *J Immunol* (2016) 197(12):4791–806. doi: 10.4049/jimmunol.1600672
19. Nutt SL, Chopin M. Transcriptional Networks Driving Dendritic Cell Differentiation and Function. *Immunity* (2020) 52(6):942–56. doi: 10.1016/j.immuni.2020.05.005
20. Ah Kioon MD, Tripodo C, Fernandez D, Kirou KA, Spiera RF, Crow MK, et al. Plasmacytoid dendritic cells promote systemic sclerosis with a key role for TLR8. *Sci Transl Med* (2018) 10(423):eaam8458. doi: 10.1126/scitranslmed.aam8458
21. Rowland SL, Riggs JM, Gilfillan S, Bugatti M, Vermi W, Kolbeck R, et al. Early, transient depletion of plasmacytoid dendritic cells ameliorates autoimmunity in a lupus model. *J Exp Med* (2014) 211(10):1977–91. doi: 10.1084/jem.20132620
22. Sisirak V, Ganguly D, Lewis KL, Couillault C, Tanaka L, Bolland S, et al. Genetic evidence for the role of plasmacytoid dendritic cells in systemic lupus erythematosus. *J Exp Med* (2014) 211(10):1969–76. doi: 10.1084/jem.20132522
23. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol* (2015) 15(8):471–85. doi: 10.1038/nri3865
24. Swiecki M, Gilfillan S, Vermi W, Wang Y, Colonna M. Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. *Immunity* (2010) 33(6):955–66. doi: 10.1016/j.immuni.2010.11.020
25. Gordon JR. TGFbeta1 and TNFalpha secreted by mast cells stimulated via the FcepsilonRI activate fibroblasts for high-level production of monocyte chemoattractant protein-1 (MCP-1). *Cell Immunol* (2000) 201(1):42–9. doi: 10.1006/cimm.2000.1631
26. Liu K, Waskow C, Liu X, Yao K, Hoh J, Nussenzweig M. Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol* (2007) 8(6):578–83. doi: 10.1038/ni1462
27. Fucikova J, Palova-Jelinkova L, Bartunkova J, Spisek R. Induction of Tolerance and Immunity by Dendritic Cells: Mechanisms and Clinical Applications. *Front Immunol* (2019) 10:2393. doi: 10.3389/fimmu.2019.02393
28. Collin M, Bigley V. Human dendritic cell subsets: an update. *Immunology* (2018) 154(1):3–20. doi: 10.1111/imm.12888
29. Musumeci A, Lutz K, Winheim E, Krug AB. What Makes a pDC: Recent Advances in Understanding Plasmacytoid DC Development and Heterogeneity. *Front Immunol* (2019) 10:1222. doi: 10.3389/fimmu.2019.01222
30. Rhodes JW, Tong O, Harman AN, Turville SG. Human Dendritic Cell Subsets, Ontogeny, and Impact on HIV Infection. *Front Immunol* (2019) 10:1088. doi: 10.3389/fimmu.2019.01088
31. Ito T, Wang YH, Duramad O, Hanabuchi S, Perng OA, Gilliet M, et al. OX40 ligand shuts down IL-10-producing regulatory T cells. *Proc Natl Acad Sci U S A* (2006) 103(35):13138–43. doi: 10.1073/pnas.0603107103
32. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* (1992) 176(6):1693–702. doi: 10.1084/jem.176.6.1693
33. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* (2000) 18:767–811. doi: 10.1146/annurev.immunol.18.1.767
34. Steinbrink K, Mahnke K, Grabbe S, Enk AH, Jonuleit H. Myeloid dendritic cell: From sentinel of immunity to key player of peripheral tolerance? *Hum Immunol* (2009) 70(5):289–93. doi: 10.1016/j.humimm.2009.02.003
35. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* (2001) 2(8):725–31. doi: 10.1038/90667
36. Faria AM, Weiner HL. Oral tolerance. *Immunol Rev* (2005) 206:232–59. doi: 10.1111/j.0105-2896.2005.00280.x
37. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med* (2008) 205(9):2139–49. doi: 10.1084/jem.20080414
38. Yokota A, Takeuchi H, Maeda N, Ohoka Y, Kato C, Song SY, et al. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int Immunol* (2009) 21(4):361–77. doi: 10.1093/intimm/dxp003
39. Steptoe RJ, Thomson AW. Dendritic cells and tolerance induction. *Clin Exp Immunol* (1996) 105(3):397–402. doi: 10.1046/j.1365-2249.1996.d01-779.x
40. Ma Y, Dawicki W, Zhang X, Gordon JR. Contributions of direct versus indirect mechanisms for regulatory dendritic cell suppression of asthmatic allergen-specific IgG1 antibody responses. *PloS One* (2018) 13(1):e0190414. doi: 10.1371/journal.pone.0190414
41. Boldison J, Da Rosa LC, Davies J, Wen L, Wong FS. Dendritic cells license regulatory B cells to produce IL-10 and mediate suppression of antigen-specific CD8 T cells. *Cell Mol Immunol* (2020) 17(8):843–55. doi: 10.1038/s41423-019-0324-z
42. Garcia-Gonzalez P, Ubilla-Olguin G, Catalan D, Schinnerling K, Aguillon JC. Tolerogenic dendritic cells for reprogramming of lymphocyte responses in autoimmune diseases. *Autoimmun Rev* (2016) 15(11):1071–80. doi: 10.1016/j.autrev.2016.07.032
43. Audiger C, Rahman MJ, Yun TJ, Tarbell KV, Lesage S. The Importance of Dendritic Cells in Maintaining Immune Tolerance. *J Immunol* (2017) 198 (6):2223–31. doi: 10.4049/jimmunol.1601629
44. Bourque J, Hawiger D. Immunomodulatory Bonds of the Partnership between Dendritic Cells and T Cells. *Crit Rev Immunol* (2018) 38(5):379–401. doi: 10.1615/CritRevImmunol.2018026790
45. Esebanmen GE, Langridge WHR. The role of TGF-beta signaling in dendritic cell tolerance. *Immunol Res* (2017) 65(5):987–94. doi: 10.1007/s12026-017-8944-9
46. Iberg CA, Hawiger D. Natural and Induced Tolerogenic Dendritic Cells. *J Immunol* (2020) 204(4):733–44. doi: 10.4049/jimmunol.1901121
47. Iberg CA, Jones A, Hawiger D. Dendritic Cells As Inducers of Peripheral Tolerance. *Trends Immunol* (2017) 38(11):793–804. doi: 10.1016/j.it.2017.07.007
48. Zanoni I, Granucci F, Foti M, Ricciardi-Castagnoli P. Self-tolerance, dendritic cell (DC)-mediated activation and tissue distribution of natural killer (NK) cells. *Immunol Lett* (2007) 110(1):6–17. doi: 10.1016/j.imlet.2007.03.001
49. Elizondo DM, Andargie TE, Haddock NL, da Silva RLL, de Moura TR, Lipscomb MW. IL-10 producing CD8(+) CD122(+) PD-1(+) regulatory T cells are expanded by dendritic cells silenced for Allograft Inflammatory Factor-1. *J Leukoc Biol* (2019) 105(1):123–30. doi: 10.1002/JLB.1A0118-010RR
50. Gordon JR, Ma Y, Churchman L, Gordon SA, Dawicki W, et al. Regulatory dendritic cells for immunotherapy in immunologic diseases. *Front Immunol* (2014) 5:7. doi: 10.3389/fimmu.2014.00007
51. Gordon JR, Li F, Nayyar A, Xiang J, Zhang X, et al. CD8 alpha+, but not CD8 alpha-, dendritic cells tolerize Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. *J Immunol* (2005) 175 (3):1516–22. doi: 10.4049/jimmunol.175.3.1516
52. Huang YS, Ogbechi J, Clanchy FI, Williams RO, Stone TW, Disorders CNS, et al. IDO and Kynurenine Metabolites in Peripheral and CNS Disorders. *Front Immunol* (2020) 11:388. doi: 10.3389/fimmu.2020.00388
53. Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, et al. T cell apoptosis by tryptophan catabolism. *Cell Death Differ* (2002) 9 (10):1069–77. doi: 10.1038/sj.cdd.4401073

54. Suss G, Shortman K. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J Exp Med* (1996) 183(4):1789–96. doi: 10.1084/jem.183.4.1789
55. Xu X, Yi H, Guo Z, Qian C, Xia S, Yao Y, et al. Splenic stroma-educated regulatory dendritic cells induce apoptosis of activated CD4 T cells via Fas ligand-enhanced IFN-gamma and nitric oxide. *J Immunol* (2012) 188 (3):1168–77. doi: 10.4049/jimmunol.1101696
56. Li XL, Yang AM, Huang H, Zhang XB, Town J, Davis B, et al. Induction of Type 2 T helper cell allergen tolerance by IL-10-differentiated regulatory dendritic cells. *Am J Resp Cell Mol Biol* (2010) 42(2):190–9. doi: 10.1165/rcmb.2009-0023OC
57. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* (2010) 116 (6):935–44. doi: 10.1182/blood-2009-07-234872
58. LeMaoult J, Krawice-Radanne I, Dausset J, Carosella ED. HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4+ T cells. *Proc Natl Acad Sci U S A* (2004) 101(18):7064–9. doi: 10.1073/pnas.0401922101
59. Amodio G, Comi M, Tomasoni D, Gianolini ME, Rizzo R, LeMaoult J, et al. HLA-G expression levels influence the tolerogenic activity of human DC-10. *Haematologica* (2015) 100(4):548–57. doi: 10.3324/haematol.2014.113803
60. Svajger U, Rozman PJ. Synergistic Effects of Interferon-gamma and Vitamin D3 Signaling in Induction of ILT-3(high)PDL-1(high) Tolerogenic Dendritic Cells. *Front Immunol* (2019) 10:2627. doi: 10.3389/fimmu.2019.02627
61. Song SS, Yuan PF, Wu HX, Chen JY, Fu JJ, Li PP, et al. Dendritic cells with an increased PD-L1 by TGF-beta induce T cell anergy for the cytotoxicity of hepatocellular carcinoma cells. *Int Immunopharmacol* (2014) 20(1):117–23. doi: 10.1016/j.intimp.2014.02.027
62. Zahorchak AF, Macedo C, Hamm DE, Butterfield LH, Metes DM, Thomson AW, et al. High PD-L1/CD86 MFI ratio and IL-10 secretion characterize human regulatory dendritic cells generated for clinical testing in organ transplantation. *Cell Immunol* (2018) 323:9–18. doi: 10.1016/j.cellimm.2017.08.008
63. Saul L, Mair I, Ivens A, Brown P, Samuel K, Campbell JDM, et al. 1,25-Dihydroxyvitamin D3 Restrains CD4(+) T Cell Priming Ability of CD11c(+) Dendritic Cells by Upregulating Expression of CD31. *Front Immunol* (2019) 10:600. doi: 10.3389/fimmu.2019.00600
64. Clement M, Fornasa G, Guedj K, Ben Mkaddem S, Gaston AT, Khallou-Laschet J, et al. CD31 is a key coinhibitory receptor in the development of immunogenic dendritic cells. *Proc Natl Acad Sci U S A* (2014) 111(12): E1101–10. doi: 10.1073/pnas.1314505111
65. Schulke S. Induction of Interleukin-10 Producing Dendritic Cells As a Tool to Suppress Allergen-Specific T Helper 2 Responses. *Front Immunol* (2018) 9:455. doi: 10.3389/fimmu.2018.00455
66. Ouyang W, O'Garra A. IL-10 Family Cytokines IL-10 and IL-22: from Basic Science to Clinical Translation. *Immunity* (2019) 50(4):871–91. doi: 10.1016/j.immuni.2019.03.020
67. Huang H, Dawicki W, Lu M, Nayyar A, Zhang X, Gordon JR. Regulatory dendritic cell expression of MHCII and IL-10 are jointly requisite for induction of tolerance in a murine model of OVA-asthma. *Allergy* (2013) 68(9):1126–35. doi: 10.1111/all.12203
68. Koya T, Matsuda H, Takeda K, Matsubara S, Miyahara N, Balhorn A, et al. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. *J Allergy Clin Immunol* (2007) 119(5):1241–50. doi: 10.1016/j.jaci.2007.01.039
69. Lu M, Dawicki W, Zhang X, Huang H, Nayyar A, Gordon JR. Therapeutic induction of tolerance by IL-10-differentiated dendritic cells in a mouse model of house dust mite-asthma. *Allergy* (2011) 66(5):612–20. doi: 10.1111/j.1398-9995.2010.02526.x
70. Nayyar A, Dawicki W, Huang H, Lu MP, Zhang XB, Gordon JR. Induction of prolonged asthma tolerance by IL-10-differentiated dendritic cells: differential impact on airway hyperresponsiveness and the Th2 immunoinflammatory response. *J Immunol* (2012) 189(1):72–9. doi: 10.4049/jimmunol.1103286
71. Coomes SM, Kannan Y, Pelly VS, Entwistle LJ, Guidi R, Perez-Lloret J, et al. CD4(+) Th2 cells are directly regulated by IL-10 during allergic airway inflammation. *Mucosal Immunol* (2017) 10(1):150–61. doi: 10.1038/mi.2016.47
72. Batlle E, Massague J. Transforming Growth Factor-beta Signaling in Immunity and Cancer. *Immunity* (2019) 50(4):924–40. doi: 10.1016/j.immuni.2019.03.024
73. Anderson AE, Swan DJ, Wong OY, Buck M, Eltherington O, Harry RA, et al. Tolerogenic dendritic cells generated with dexamethasone and vitamin D3 regulate rheumatoid arthritis CD4(+) T cells partly via transforming growth factor-beta1. *Clin Exp Immunol* (2017) 187(1):113–23. doi: 10.1111/cei.12870
74. Dawicki W, Li CY, Town J, Zhang XB, Gordon JR. Therapeutic reversal of food allergen sensitivity by mature retinoic acid-differentiated dendritic cell induction of LAG3(+)CD49b(-)Foxp3(-) regulatory T cells. *J Allergy Clin Immunol* (2017) 139(5):1608–20. doi: 10.1016/j.jaci.2016.07.042
75. Qi J, Zhang Z, Tang X, Li W, Chen W, Yao G. IL-27 Regulated CD4(+)IL-10(+) T Cells in Experimental Sjogren Syndrome. *Front Immunol* (2020) 11:1699. doi: 10.3389/fimmu.2020.01699
76. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* (2009) 10 (7):689–95. doi: 10.1038/ni.1760
77. Huang H, Dawicki W, Zhang XB, Town J, Gordon JR. Tolerogenic dendritic cells induce CD4(+)CD25(hi)Foxp3(+) regulatory T cell differentiation from CD4(+)CD25(-/lo)Foxp3(-) effector T cells. *J Immunol* (2010) 185(9):5003–10. doi: 10.4049/jimmunol.0903446
78. Huang H, Ma Y, Dawicki W, Zhang X, Gordon JR. Comparison of induced versus natural regulatory T cells of the same TCR specificity for induction of tolerance to an environmental antigen. *J Immunol* (2013) 191(3):1136–43. doi: 10.4049/jimmunol.1201899
79. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M, Gagliani N, et al. The Biology of T Regulatory Type 1 Cells and Their Therapeutic Application in Immune-Mediated Diseases. *Immunity* (2018) 49(6):1004–19. doi: 10.1016/j.immuni.2018.12.001
80. Carrier Y, Yuan J, Kuchroo VK, Weiner HL. Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. *J Immunol* (2007) 178 (1):179–85. doi: 10.4049/jimmunol.178.1.179
81. Du X, Chang S, Guo W, Zhang S, Chen ZK. Progress in Liver Transplant Tolerance and Tolerance-Inducing Cellular Therapies. *Front Immunol* (2020) 11:1326. doi: 10.3389/fimmu.2020.01326
82. Thomson AW, Zahorchak AF, Ezzelarab MB, Butterfield LH, Lakkis FG, Metes DM. Prospective Clinical Testing of Regulatory Dendritic Cells in Organ Transplantation. *Front Immunol* (2016) 7:15. doi: 10.3389/fimmu.2016.00015
83. Ritprajak P, Kaewraemruaen C, Hirankarn N. Current Paradigms of Tolerogenic Dendritic Cells and Clinical Implications for Systemic Lupus Erythematosus. *Cells* (2019) 8(10):1291. doi: 10.3390/cells8101291
84. Pellerin L, Jenks JA, Chinthrajah S, Dominguez T, Block W, Zhou X, et al. Peanut-specific type 1 regulatory T cells induced in vitro from allergic subjects are functionally impaired. *J Allergy Clin Immunol* (2017) 141:202–13. doi: 10.1016/j.jaci.2017.05.045
85. Ruiter B, Smith NP, Fleming E, Patil SU, Hurlburt BK, Maleki SJ, et al. Peanut protein acts as a Th2 adjuvant by inducing RALDH2 in human antigen-presenting cells. *J Allergy Clin Immunol* (2021). doi: 10.1016/j.jaci.2020.11.047
86. Garin MI, Chu CC, Golshayan D, Cernuda-Morollon E, Wait R, Lechler RI. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* (2007) 109(5):2058–65. doi: 10.1182/blood-2006-04-016451
87. Okeke EB, Uzonna JE. The Pivotal Role of Regulatory T Cells in the Regulation of Innate Immune Cells. *Front Immunol* (2019) 10:680. doi: 10.3389/fimmu.2019.00680
88. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* (2009) 30(5):636–45. doi: 10.1016/j.immuni.2009.04.010
89. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* (2006) 6(4):295–307. doi: 10.1038/nri1806
90. Kanamori M, Nakatsukasa H, Okada M, Lu Q, Yoshimura A. Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends Immunol* (2016) 37(11):803–11. doi: 10.1016/j.it.2016.08.012
91. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity* (2013) 38(3):414–23. doi: 10.1016/j.immuni.2013.03.002

92. Gravano DM, Vignali DA. The battle against immunopathology: infectious tolerance mediated by regulatory T cells. *Cell Mol Life Sci* (2012) 69 (12):1997–2008. doi: 10.1007/s00018-011-0907-z
93. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol* (2008) 180(9):5916–26. doi: 10.4049/jimmunol.180.9.5916
94. Awasthi A, Carrier Y, Peron JP, Bettelli E, Kamanaka M, Flavell RA, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* (2007) 8(12):1380–9. doi: 10.1038/ni1541
95. Ilarregui JM, Croci DO, Bianco GA, Toscano MA, Salatino M, Vermeulen ME, et al. Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nat Immunol* (2009) 10(9):981–91. doi: 10.1038/ni.1772
96. Carrier Y, Yuan J, Kuchroo VK, Weiner HL. Th3 cells in peripheral tolerance. II. TGF-beta-transgenic Th3 cells rescue IL-2-deficient mice from autoimmunity. *J Immunol* (2007) 178(1):172–8. doi: 10.4049/jimmunol.178.1.172
97. Andersson J, Tran DQ, Pesu M, Davidson TS, Ramsey H, O'Shea JJ, et al. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J Exp Med* (2008) 205(9):1975–81. doi: 10.1084/jem.20080308
98. Kim SH, Jung HH, Lee CK. Generation, Characteristics and Clinical Trials of Ex Vivo Generated Tolerogenic Dendritic Cells. *Yonsei Med J* (2018) 59 (7):807–15. doi: 10.3349/ymj.2018.59.7.807
99. Nair S, Archer GE, Tedder TF. Isolation and generation of human dendritic cells. *Curr Protoc Immunol* (2012) Chapter 7:Unit7 32. doi: 10.1002/0471142735.im0732s99
100. Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, et al. Proliferating dendritic cell progenitors in human blood. *J Exp Med* (1994) 180(1):83–93. doi: 10.1084/jem.180.1.83
101. Zhang J, Supakorndej T, Krambs JR, Rao M, Abou-Ezzi G, Ye RY, et al. Bone marrow dendritic cells regulate hematopoietic stem/progenitor cell trafficking. *J Clin Invest* (2019) 129(7):2920–31. doi: 10.1172/JCI124829
102. Sachamitr P, Leishman AJ, Davies TJ, Fairchild PJ. Directed Differentiation of Human Induced Pluripotent Stem Cells into Dendritic Cells Displaying Tolerogenic Properties and Resembling the CD141(+) Subset. *Front Immunol* (2017) 8:1935. doi: 10.3389/fimmu.2017.01935
103. Baghbaderani BA, Tian X, Neo BH, Burkall A, Dimezzo T, Sierra G, et al. cGMP-Manufactured Human Induced Pluripotent Stem Cells Are Available for Pre-clinical and Clinical Applications. *Stem Cell Rep* (2015) 5(4):647–59. doi: 10.1016/j.stemcr.2015.08.015
104. Choi KD, Vodyanik MA, Slukvin II. Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors. *J Clin Invest* (2009) 119(9):2818–29. doi: 10.1172/JCI38591
105. Senju S, Haruta M, Matsumura K, Matsunaga Y, Fukushima S, Ikeda T, et al. Generation of dendritic cells and macrophages from human induced pluripotent stem cells aiming at cell therapy. *Gene Ther* (2011) 18(9):874–83. doi: 10.1038/gt.2011.22
106. Goriely S, Van Lint C, Dadkhah R, Libin M, De Wit D, Demonte D, et al. A defect in nucleosome remodeling prevents IL-12(p35) gene transcription in neonatal dendritic cells. *J Exp Med* (2004) 199(7):1011–6. doi: 10.1084/jem.20031272
107. Goriely S, Vincart B, Stordeur P, Vekemans J, Willems F, Goldman M, et al. Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes. *J Immunol* (2001) 166(3):2141–6. doi: 10.4049/jimmunol.166.3.2141
108. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, et al. Epigenetic memory in induced pluripotent stem cells. *Nature* (2010) 467(7313):285–90. doi: 10.1038/nature09342
109. Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, et al. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotechnol* (2010) 28(8):848–55. doi: 10.1038/nbt.1667
110. Schmidt SV, Nino-Castro AC, Schultze JL. Regulatory dendritic cells: there is more than just immune activation. *Front Immunol* (2012) 3:274. doi: 10.3389/fimmu.2012.00274
111. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* (1998) 392(6673):245–52. doi: 10.1038/32588
112. Horton C, Shanmugarajah K, Fairchild PJ. Harnessing the properties of dendritic cells in the pursuit of immunological tolerance. *BioMed J* (2017) 40 (2):80–93. doi: 10.1016/j.bj.2017.01.002
113. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell* (2001) 106(3):255–8. doi: 10.1016/S0092-8674(01)00449-4
114. Yamazaki S, Bonito AJ, Spisek R, Dhodapkar M, Inaba K, Steinman RM. Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors. *Blood* (2007) 110(13):4293–302. doi: 10.1182/blood-2007-05-088831
115. Nguyen QT, Jang E, Le HT, Kim S, Kim D, Dvorina N, et al. IL-27 targets Foxp3+ Tregs to mediate antiinflammatory functions during experimental allergic airway inflammation. *JCI Insight* (2019) 4(2):e123216. doi: 10.1172/jci.insight.123216
116. Peng Q, Qiu X, Zhang Z, Zhang S, Zhang Y, Liang Y, et al. PD-L1 on dendritic cells attenuates T cell activation and regulates response to immune checkpoint blockade. *Nat Commun* (2020) 11(1):4835. doi: 10.1038/s41467-020-18570-x
117. Sun W, Wei JW, Li H, Wei FQ, Li J, Wen WP, et al. Adoptive cell therapy of tolerogenic dendritic cells as inducer of regulatory T cells in allergic rhinitis. *Int Forum Allergy Rhinol* (2018) 8(11):1291–9. doi: 10.1002/alr.22217
118. Krakow S, Crescimone ML, Bartels C, Wiegering V, Eyrich M, Schlegel PG, et al. Re-expression of CD14 in Response to a Combined IL-10/TLR Stimulus Defines Monocyte-Derived Cells With an Immunoregulatory Phenotype. *Front Immunol* (2019) 10:1484. doi: 10.3389/fimmu.2019.01484
119. Kryczanowsky F, Raker V, Graulich E, Domogalla MP, Steinbrink K. IL-10-Modulated Human Dendritic Cells for Clinical Use: Identification of a Stable and Migratory Subset with Improved Tolerogenic Activity. *J Immunol* (2016) 197(9):3607–17. doi: 10.4049/jimmunol.1501769
120. Van Overtvelt L, Lombardi V, Razafindratsita A, Saint-Lu N, Horiot S, Moussu H, et al. IL-10-inducing Adjuvants enhance sublingual immunotherapy efficacy in a murine asthma model. *Int Arch Allergy Immunol* (2008) 145(2):152–62. doi: 10.1159/000108140
121. Yurchenko E, Shio MT, Huang TC, Da Silva Martins M, Szyf M, Levings MK, et al. Inflammation-driven reprogramming of CD4+ Foxp3+ regulatory T cells into pathogenic Th1/Th17 T effectors is abrogated by mTOR inhibition in vivo. *PloS One* (2012) 7(4):e35572. doi: 10.1371/journal.pone.0035572
122. Lim DS, Kang MS, Jeong JA, Bae YS. Semi-mature DC are immunogenic and not tolerogenic when inoculated at a high dose in collagen-induced arthritis mice. *Eur J Immunol* (2009) 39(5):1334–43. doi: 10.1002/eji.200838987
123. Voigtlander C, Rossner S, Cierpka E, Theiner G, Wiethe C, Menges M, et al. Dendritic cells matured with TNF can be further activated in vitro and after subcutaneous injection in vivo which converts their tolerogenicity into immunogenicity. *J Immunother* (2006) 29(4):407–15. doi: 10.1097/01.cji.0000210081.60178.b4
124. Engman C, Garciafigueroa Y, Phillips BE, Trucco M, Giannoukakis N. Co-Stimulation-Impaired Bone Marrow-Derived Dendritic Cells Prevent Dextran Sodium Sulfate-Induced Colitis in Mice. *Front Immunol* (2018) 9:894. doi: 10.3389/fimmu.2018.00894
125. Vanherwegen AS, Cook DP, Ferreira GB, Gysemans C, Mathieu C, et al. Vitamin D-modulated dendritic cells delay lethal graft-versus-host disease through induction of regulatory T cells. *J Steroid Biochem Mol Biol* (2019) 188:103–10. doi: 10.1016/j.jsbmb.2018.12.013
126. Penna G, Roncari A, Amuchastegui S, Daniel KC, Berti E, Colonna M, et al. Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+ regulatory T cells by 1,25-dihydroxyvitamin D3. *Blood* (2005) 106(10):3490–7. doi: 10.1182/blood-2005-05-2044
127. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol* (2009) 39(11):3147–59. doi: 10.1002/eji.200839103
128. van der Aar AM, Sibiryak DS, Bakdash G, van Capel TM, van der Kleij HP, Opstelten DJ, et al. Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. *J Allergy Clin Immunol* (2011) 127 (6):1532–40.e7. doi: 10.1016/j.jaci.2011.01.068

129. Kraus LF, Scheurmann N, Frenzel DF, Tasdogan A, Weiss JM. 9-cis-Retinoic acid induces a distinct regulatory dendritic cell phenotype that modulates murine delayed-type allergy. *Contact Dermatitis* (2018) 78(1):41–54. doi: 10.1111/cod.12868
130. Elizondo DM, Andargie TE, Yang D, Kacsinta AD, Lipscomb MW. Inhibition of Allograft Inflammatory Factor-1 in Dendritic Cells Restrains CD4(+) T Cell Effector Responses and Induces CD25(+)Foxp3(+) T Regulatory Subsets. *Front Immunol* (2017) 8:1502. doi: 10.3389/fimmu.2017.01502
131. Jimenez RV, Wright TT, Jones NR, Wu J, Gibson AW, Szalai AJ. C-Reactive Protein Impairs Dendritic Cell Development, Maturation, and Function: Implications for Peripheral Tolerance. *Front Immunol* (2018) 9:372. doi: 10.3389/fimmu.2018.00372
132. Suzuki M, Yokota M, Kanemitsu Y, Min WP, Ozaki S, Nakamura Y. Intranasal administration of regulatory dendritic cells is useful for the induction of nasal mucosal tolerance in a mice model of allergic rhinitis. *World Allergy Organ J* (2020) 13(8):100447. doi: 10.1016/j.waojou.2020.100447
133. Santos ES, de Aragao-Franca LS, Meira CS, Cerqueira JV, Vasconcelos JF, Nonaka CKV, et al. Tolerogenic Dendritic Cells Reduce Cardiac Inflammation and Fibrosis in Chronic Chagas Disease. *Front Immunol* (2020) 11:488. doi: 10.3389/fimmu.2020.00488
134. de Aragao-Franca LS, Aragao-Franca LS, Rocha VCJ, Rocha VCJ, Cronemberger-Andrade A, da Costa FHB, et al. Tolerogenic Dendritic Cells Reduce Airway Inflammation in a Model of Dust Mite Triggered Allergic Inflammation. *Allergy Asthma Immunol Res* (2018) 10(4):406–19. doi: 10.4168/aair.2018.10.4.406
135. Lynch K, Treacy O, Gerlach JQ, Annuk H, Lohan P, Cabral J, et al. Regulating Immunogenicity and Tolerogenicity of Bone Marrow-Derived Dendritic Cells through Modulation of Cell Surface Glycosylation by Dexamethasone Treatment. *Front Immunol* (2017) 8:1427. doi: 10.3389/fimmu.2017.01427
136. Kim N, Park CS, Im SA, Kim JW, Lee JH, Park YJ, et al. Minocycline promotes the generation of dendritic cells with regulatory properties. *Oncotarget* (2016) 7(33):52818–31. doi: 10.18632/oncotarget.10810
137. Lee JH, Park CS, Jang S, Kim JW, Kim SH, Song S, et al. Tolerogenic dendritic cells are efficiently generated using minocycline and dexamethasone. *Sci Rep* (2017) 7(1):15087. doi: 10.1038/s41598-017-15569-1
138. Wan J, Huang F, Hao S, Hu W, Liu C, Zhang W, et al. Interleukin-10 Gene-Modified Dendritic Cell-Induced Type 1 Regulatory T Cells Induce Transplant-Tolerance and Impede Graft Versus Host Disease After Allogeneic Stem Cell Transplantation. *Cell Physiol Biochem* (2017) 43(1):353–66. doi: 10.1159/000480415
139. Wu H, Lo Y, Chan A, Law KS, Mok MY. Rel B-modified dendritic cells possess tolerogenic phenotype and functions on lupus splenic lymphocytes in vitro. *Immunology* (2016) 149(1):48–61. doi: 10.1111/imm.12628
140. Wu H, Xu Z, Wang Z, Ren Z, Li L, Ruan Y. Dendritic cells with METTL3 gene knockdown exhibit immature properties and prolong allograft survival. *Genes Immun* (2020) 21(3):193–202. doi: 10.1038/s41435-020-0099-3
141. Jansen MAA, Spiering R, Ludwig IS, van Eden W, Hilkens CMU, Broere F. Matured Tolerogenic Dendritic Cells Effectively Inhibit Autoantigen Specific CD4(+) T Cells in a Murine Arthritis Model. *Front Immunol* (2019) 10:2068. doi: 10.3389/fimmu.2019.02068
142. Ezzelarab MB, Raich-Regue D, Lu L, Zahorchak AF, Perez-Gutierrez A, Humar A, et al. Renal Allograft Survival in Nonhuman Primates Infused With Donor Antigen-Pulsed Autologous Regulatory Dendritic Cells. *Am J Transplant* (2017) 17(6):1476–89. doi: 10.1111/ajt.14182
143. Zahorchak AF, Kean LS, Tokita D, Turnquist HR, Abe M, Finke J, et al. Infusion of stably immature monocyte-derived dendritic cells plus CTLA4Ig modulates alloimmune reactivity in rhesus macaques. *Transplantation* (2007) 84(2):196–206. doi: 10.1097/01.tp.0000268582.21168.f6
144. Zahorchak AF, Raimondi G, Thomson AW. Rhesus monkey immature monocyte-derived dendritic cells generate alloantigen-specific regulatory T cells from circulating CD4+CD127-/lo T cells. *Transplantation* (2009) 88(9):1057–64. doi: 10.1097/TP.0b013e3181ba6b1f
145. Ezzelarab MB, Lu L, Shufesky WF, Morelli AE, Thomson AW. Donor-Derived Regulatory Dendritic Cell Infusion Maintains Donor-Reactive CD4(+)CTLA4(hi) T Cells in Non-Human Primate Renal Allograft Recipients Treated with CD28 Co-Stimulation Blockade. *Front Immunol* (2018) 9:250. doi: 10.3389/fimmu.2018.00250
146. Ezzelarab MB, Zahorchak AF, Lu L, Morelli AE, Chalasani G, Demetris AJ, et al. Regulatory dendritic cell infusion prolongs kidney allograft survival in nonhuman primates. *Am J Transplant* (2013) 13(8):1989–2005. doi: 10.1111/ajt.12310
147. Wang G, Zhang J, Fang Y, Cao W, Xu B, Chen X. Stimulation of tolerogenic dendritic cells using dexamethasone and 1,25-dihydroxyvitamin D3 represses autologous T cell activation and chondrocyte inflammation. *Exp Ther Med* (2019) 17(1):679–88. doi: 10.3892/etm.2018.7036
148. Djedovic N, Mansilla MJ, Jevtic B, Navarro-Barriuso J, Saksida T, Martinez-Caceres EM, et al. Ethyl Pyruvate Induces Tolerogenic Dendritic Cells. *Front Immunol* (2019) 10:157. doi: 10.3389/fimmu.2019.00157
149. Comi M, Amodio G, Passeri L, Fortunato M, Santoni de Sio FR, Andolfi G, et al. Generation of Powerful Human Tolerogenic Dendritic Cells by Lentiviral-Mediated IL-10 Gene Transfer. *Front Immunol* (2020) 11:1260. doi: 10.3389/fimmu.2020.01260
150. Agrawal S, Ganguly S, Tran A, Sundaram P, Agrawal A. Retinoic acid treated human dendritic cells induce T regulatory cells via the expression of CD141 and GARP which is impaired with age. *Aging (Albany NY)* (2016) 8(6):1223–35. doi: 10.18632/aging.100973
151. Richardson JR, Armbruster NS, Gunter M, Henes J, Autenrieth SE, et al. Staphylococcus aureus PSM Peptides Modulate Human Monocyte-Derived Dendritic Cells to Prime Regulatory T Cells. *Front Immunol* (2018) 9:2603. doi: 10.3389/fimmu.2018.02603
152. Sato K, Yamashita N, Matsuyama T. Human peripheral blood monocyte-derived interleukin-10-induced semi-mature dendritic cells induce anergic CD4(+) and CD8(+) T cells via presentation of the internalized soluble antigen and cross-presentation of the phagocytosed necrotic cellular fragments. *Cell Immunol* (2002) 215(2):186–94. doi: 10.1016/S0008-8749(02)00021-7
153. Enk AH, Jonuleit H, Saloga J, Knop J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int J Cancer* (1997) 73(3):309–16. doi: 10.1002/(SICI)1097-0215(19971104)73:3<309::AID-IJC1>3.0.CO;2-3
154. Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* (1997) 159(10):4772–80.
155. Bellinghausen I, Brand U, Steinbrink K, Enk AH, Knop J, Saloga J, et al. Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. *J Allergy Clin Immunol* (2001) 108(2):242–9. doi: 10.1067/mai.2001.117177
156. Boks MA, Kager-Groenland JR, Haasjes MS, Zwaginga JJ, van Ham SM, ten Brinke A. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction–a comparative study of human clinical-applicable DC. *Clin Immunol* (2012) 142(3):332–42. doi: 10.1016/j.clim.2011.11.011
157. Chen L, Qiu M, He W, Huang A, Liu J. Functional study of immature dendritic cells co-transfected with IL-10 and TGF-beta 1 genes in vitro. *Mol Biol Rep* (2012) 39(6):6633–9. doi: 10.1007/s11033-012-1468-4
158. Figueroa-Vega N, Galindo-Rodriguez G, Bajana S, Portales-Perez D, Abud-Mendoza C, Sanchez-Torres C, et al. Phenotypic analysis of IL-10-treated, monocyte-derived dendritic cells in patients with systemic lupus erythematosus. *Scand J Immunol* (2006) 64(6):668–76. doi: 10.1111/j.1365-3083.2006.01849.x
159. Henry E, Desmet CJ, Garze V, Fievez L, Bedoret D, Heirman C, et al. Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma. *J Immunol* (2008) 181(10):7230–42. doi: 10.4049/jimmunol.181.10.7230
160. Menges M, Rossner S, Voigtlander C, Schindler H, Kukutsch NA, Bogdan C, et al. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J Exp Med* (2002) 195(1):15–21. doi: 10.1084/jem.20011341
161. Sato K, Yamashita N, Baba M, Matsuyama T. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood* (2003) 101(9):3581–9. doi: 10.1182/blood-2002-09-2712
162. Rutella S, Bonanno G, Procoli A, Mariotti A, de Ritis DG, Curti A, et al. Hepatocyte growth factor favors monocyte differentiation into regulatory

interleukin (IL)-10++IL-12low/neg accessory cells with dendritic-cell features. *Blood* (2006) 108(1):218–27. doi: 10.1182/blood-2005-08-3141
163. Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* (2006) 108(5):1435–40. doi: 10.1182/blood-2006-03-006403
164. Torres-Aguilar H, Aguilar-Ruiz SR, Gonzalez-Perez G, Munguia R, Bajana S, Meraz-Rios MA, et al. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4+ T cells. *J Immunol* (2010) 184(4):1765–75. doi: 10.4049/jimmunol.0902133
165. Zhang-Hoover J, Finn P, Stein-Streilein J. Modulation of ovalbumin-induced airway inflammation and hyperreactivity by tolerogenic APC. *J Immunol* (2005) 175(11):7117–24. doi: 10.4049/jimmunol.175.11.7117
166. Piemonti L, Monti P, Allavena P, Sironi M, Soldini L, Leone BE, et al. Glucocorticoids affect human dendritic cell differentiation and maturation. *J Immunol* (1999) 162(11):6473–81.
167. Naranjo-Gomez M, Raich-Regue D, Onate C, Grau-Lopez L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. *J Transl Med* (2011) 9:89. doi: 10.1186/1479-5876-9-89
168. Xia CQ, Peng R, Beato F, Clare-Salzler MJ. Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity. *Scand J Immunol* (2005) 62(1):45–54. doi: 10.1111/j.1365-3083.2005.01640.x
169. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol* (2000) 164 (5):2405–11. doi: 10.4049/jimmunol.164.5.2405
170. Chamorro S, Garcia-Vallejo JJ, Unger WW, Fernandes RJ, Bruijns SC, Laban S, et al. TLR triggering on tolerogenic dendritic cells results in TLR2 up-regulation and a reduced proinflammatory immune program. *J Immunol* (2009) 183(5):2984–94. doi: 10.4049/jimmunol.0801155
171. Ferreira GB, Kleijwegt FS, Waelkens E, Lage K, Nikolic T, Hansen DA, et al. Differential protein pathways in 1,25-dihydroxyvitamin d(3) and dexamethasone modulated tolerogenic human dendritic cells. *J Proteome Res* (2012) 11(2):941–71. doi: 10.1021/pr200724e
172. Ferreira GB, van Etten E, Verstuyf A, Waer M, Overbergh L, Gysemans C, et al. 1,25-Dihydroxyvitamin D3 alters murine dendritic cell behaviour in vitro and in vivo. *Diabetes Metab Res Rev* (2011) 27(8):933–41. doi: 10.1002/dmrr.1275
173. Macedo C, Turquist H, Metes D, Thomson AW. Immunoregulatory properties of rapamycin-conditioned monocyte-derived dendritic cells and their role in transplantation. *Transplant Res* (2012) 1(1):16. doi: 10.1186/2047-1440-1-16
174. Li X, Li JJ, Yang JY, Wang DS, Zhao W, Song WJ, et al. Tolerance induction by exosomes from immature dendritic cells and rapamycin in a mouse cardiac allograft model. *PloS One* (2012) 7(8):e44045. doi: 10.1371/journal.pone.0044045
175. Gonzalez-Rey E, Chorny A, Fernandez-Martin A, Ganea D, Delgado M. Vasoactive intestinal peptide generates human tolerogenic dendritic cells that induce CD4 and CD8 regulatory T cells. *Blood* (2006) 107(9):3632–8. doi: 10.1182/blood-2005-11-4497
176. Toscano MG, Delgado M, Kong W, Martin F, Skarica M, Ganea D, et al. Dendritic cells transduced with lentiviral vectors expressing VIP differentiate into VIP-secreting tolerogenic-like DCs. *Mol Ther* (2010) 18(5):1035–45. doi: 10.1038/mt.2009.293
177. Joffre O, Nolte MA, Sporri R, Reis e Sousa C, et al. Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunol Rev* (2009) 227(1):234–47. doi: 10.1111/j.1600-065X.2008.00718.x
178. Qian C, Cao X. Dendritic cells in the regulation of immunity and inflammation. *Semin Immunol* (2018) 35:3–11. doi: 10.1016/j.smim.2017.12.002
179. Yurchenko E, Levings MK, Piccirillo CA. CD4+ Foxp3+ regulatory T cells suppress gammadelta T-cell effector functions in a model of T-cell-induced mucosal inflammation. *Eur J Immunol* (2011) 41(12):3455–66. doi: 10.1002/eji.201141814
180. Shim JU, Lee SE, Hwang W, Lee C, Park JW, Sohn JH, et al. Flagellin suppresses experimental asthma by generating regulatory dendritic cells and T cells. *J Allergy Clin Immunol* (2016) 137(2):426–35. doi: 10.1016/j.jaci.2015.07.010
181. Kim HS, Park KH, Lee HK, Kim JS, Kim YG, Lee JH, et al. Curdlan activates dendritic cells through dectin-1 and toll-like receptor 4 signaling. *Int Immunopharmacol* (2016) 39:71–8. doi: 10.1016/j.intimp.2016.07.013
182. Kawashima S, Hirose K, Iwata A, Takahashi K, Ohkubo A, Tamachi T, et al. beta-glucan curdlan induces IL-10-producing CD4+ T cells and inhibits allergic airway inflammation. *J Immunol* (2012) 189(12):5713–21. doi: 10.4049/jimmunol.1201521
183. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med* (2015) 7(307):307ra152. doi: 10.1126/scitranslmed.aab2271
184. Pujari R, Banerjee G. Impact of prebiotics on immune response: from the bench to the clinic. *Immunol Cell Biol* (2020). doi: 10.1111/imcb.12409
185. Yang J, Ren F, Zhang H, Jiang L, Hao Y, Luo X. Induction of Regulatory Dendritic Cells by Lactobacillus paracasei L9 Prevents Allergic Sensitization to Bovine beta-Lactoglobulin in Mice. *J Microbiol Biotechnol* (2015) 25 (10):1687–96. doi: 10.4014/jmb.1503.03022
186. Verma R, Lee C, Jeun EJ, Yi J, Kim KS, Ghosh A, et al. Cell surface polysaccharides of Bifidobacterium bifidum induce the generation of Foxp3(+) regulatory T cells. *Sci Immunol* (2018) 3(28):eaat6975. doi: 10.1126/sciimmunol.aat6975
187. Fu L, Song J, Wang C, Fu S, Wang Y. Bifidobacterium infantis potentially alleviates shrimp tropomyosin-induced allergy by tolerogenic dendritic cell-dependent induction of regulatory T cells and alterations in gut microbiota. *Front Immunol* (2017) 8:1536. doi: 10.3389/fimmu.2017.01536
188. Shen Y, Giardino Torchia ML, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. *Cell Host Microbe* (2012) 12 (4):509–20. doi: 10.1016/j.chom.2012.08.004
189. Durant L, Stentz R, Noble A, Brooks J, Gicheva N, Reddi D, et al. Bacteroides thetaiotaomicron-derived outer membrane vesicles promote regulatory dendritic cell responses in health but not in inflammatory bowel disease. *Microbiome* (2020) 8(1):88. doi: 10.1186/s40168-020-00868-z
190. Gudmundsdottir AB, Brynjolfsdottir A, Olafsdottir ES, Hardardottir I, Freysdottir J. Exopolysaccharides from Cyanobacterium aponinum induce a regulatory dendritic cell phenotype and inhibit SYK and CLEC7A expression in dendritic cells, T cells and keratinocytes. *Int Immunopharmacol* (2019) 69:328–36. doi: 10.1016/j.intimp.2019.01.044
191. Gudmundsdottir AB, Omarsdottir S, Brynjolfsdottir A, Paulsen BS, Olafsdottir ES, Freysdottir J, et al. Exopolysaccharides from Cyanobacterium aponinum from the Blue Lagoon in Iceland increase IL-10 secretion by human dendritic cells and their ability to reduce the IL-17 +RORgammat+/IL-10+FoxP3+ ratio in CD4+ T cells. *Immunol Lett* (2015) 163(2):157–62. doi: 10.1016/j.imlet.2014.11.008
192. Thomson AW, Ezzelarab MB. Generation and functional assessment of nonhuman primate regulatory dendritic cells and their therapeutic efficacy in renal transplantation. *Cell Immunol* (2020) 351:104087. doi: 10.1016/j.cellimm.2020.104087
193. Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M, Phase I. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* (2011) 34(9):2026–32. doi: 10.2337/dc11-0472
194. Joo YB, Park JE, Choi CB, Choi J, Jang JA, Heo M, et al. Phase 1 Study of Immunotherapy Using Autoantigen-Loaded Dendritic Cells in Patients with Anti-Citrullinated Peptide Antigen Positive Rheumatoid Arthritis. *Arthritis Rheumatol* (2014) 66:S420–1.
195. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci Trans Med* (2015) 7(290):290ra87. doi: 10.1126/scitranslmed.aaa9301
196. Jauregui-Amezaga A, Cabezon R, Ramirez-Morros A, Espana C, Rimola J, Bru C, et al. Intraperitoneal Administration of Autologous Tolerogenic Dendritic Cells for Refractory Crohn's Disease: A Phase I Study. *J Crohns Colitis* (2015) 9(12):1071–8. doi: 10.1093/ecco-jcc/jjv144
197. Bell GM, Anderson AE, Diboll J, Reece R, Eltherington O, Harry RA, et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann Rheum Dis* (2017) 76(1):227–34. doi: 10.1136/annrheumdis-2015-208456

198. Zubizarreta I, Florez-Grau G, Vila G, Cabezon R, Espana C, Andorra M, et al. Immune tolerance in multiple sclerosis and neuromyelitis optica with peptide-loaded tolerogenic dendritic cells in a phase 1b trial. *Proc Natl Acad Sci U S A* (2019) 116(17):8463–70. doi: 10.1073/pnas.1820039116
199. Macedo C, Tran LM, Zahorchak AF, Dai H, Gu X, Ravichandran R, et al. Donor-derived regulatory dendritic cell infusion results in host cell cross-dressing and T cell subset changes in prospective living donor liver transplant recipients. *Am J Transplant* (2020). doi: 10.1111/ajt.16393
200. Ten Brinke A, Martinez-Llordella M, Cools N, Hilkens CMU, van Ham SM, Sawitzki B, et al. Ways Forward for Tolerance-Inducing Cellular Therapies-an AFACTT Perspective. *Front Immunol* (2019) 10:181. doi: 10.3389/fimmu.2019.00181
201. Lundberg K, Albrekt AS, Nelissen I, Santegoets S, de Gruijl TD, Gibbs S, et al. Transcriptional profiling of human dendritic cell populations and models–unique profiles of in vitro dendritic cells and implications on functionality and applicability. *PloS One* (2013) 8(1):e52875. doi: 10.1371/journal.pone.0052875
202. Bol KF, Schreibelt G, Rabold K, Wculek SK, Schwarze JK, Dzionek A, et al. The clinical application of cancer immunotherapy based on naturally circulating dendritic cells. *J Immunother Cancer* (2019) 7(1):109. doi: 10.1186/s40425-019-0580-6
203. Adnan E, Matsumoto T, Ishizaki J, Onishi S, Suemori K, Yasukawa M, et al. Human tolerogenic dendritic cells generated with protein kinase C inhibitor are optimal for functional regulatory T cell induction - A comparative study. *Clin Immunol* (2016) 173:96–108. doi: 10.1016/j.clim.2016.09.007
204. Hasegawa H, Matsumoto T. Mechanisms of Tolerance Induction by Dendritic Cells In Vivo. *Front Immunol* (2018) 9:350. doi: 10.3389/fimmu.2018.00350
205. Funda DP, Palova-Jelinkova L, Golias J, Kroulikova Z, Fajstova A, Hudcovic T, et al. Optimal Tolerogenic Dendritic Cells in Type 1 Diabetes (T1D) Therapy: What Can We Learn From Non-obese Diabetic (NOD) Mouse Models? *Front Immunol* (2019) 10:967. doi: 10.3389/fimmu.2019.00967
206. Gueguen C, Bouley J, Moussu H, Luce S, Duchateau M, Chamot-Rooke J, et al. Changes in markers associated with dendritic cells driving the differentiation of either TH2 cells or regulatory T cells correlate with clinical benefit during allergen immunotherapy. *J Allergy Clin Immunol* (2016) 137(2):545–58. doi: 10.1016/j.jaci.2015.09.015
207. Navarro-Barriuso J, Mansilla MJ, Naranjo-Gomez M, Sanchez-Pla A, Quirant-Sanchez B, Teniente-Serra A, et al. Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3, dexamethasone and rapamycin. *Sci Rep* (2018) 8(1):14985. doi: 10.1038/s41598-018-33248-7
208. Nikolic T, Woittiez NJC, van der Slik A, Laban S, Joosten A, Gysemans C, et al. Differential transcriptome of tolerogenic versus inflammatory dendritic cells points to modulated T1D genetic risk and enriched immune regulation. *Genes Immun* (2017) 18(3):176–83. doi: 10.1038/gene.2017.18
209. Garcia-Gonzalez PA, Schinnerling K, Sepulveda-Gutierrez A, Maggi J, Mehdi AM, Nel HJ, et al. Dexamethasone and Monophosphoryl Lipid A Induce a Distinctive Profile on Monocyte-Derived Dendritic Cells through Transcriptional Modulation of Genes Associated With Essential Processes of the Immune Response. *Front Immunol* (2017) 8:1350. doi: 10.3389/fimmu.2017.01350
210. Zimmer A, Bouley J, Le Mignon M, Pliquet E, Horiot S, Turfkruyer M, et al. A regulatory dendritic cell signature correlates with the clinical efficacy of allergen-specific sublingual immunotherapy. *J Allergy Clin Immunol* (2012) 129(4):1020–30. doi: 10.1016/j.jaci.2012.02.014
211. Navarro-Barriuso J, Mansilla MJ, Martinez-Caceres EM. Searching for the Transcriptomic Signature of Immune Tolerance Induction-Biomarkers of Safety and Functionality for Tolerogenic Dendritic Cells and Regulatory Macrophages. *Front Immunol* (2018) 9:2062. doi: 10.3389/fimmu.2018.02062
212. Mascarell L, Airouche S, Berjont N, Gary C, Gueguen C, Fourcade G, et al. The regulatory dendritic cell marker C1q is a potent inhibitor of allergic inflammation. *Mucosal Immunol* (2017) 10(3):695–704. doi: 10.1038/mi.2016.87

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 25 March 2021
doi: 10.3389/fimmu.2021.589200

C-Reactive Protein Suppresses the Th17 Response Indirectly by Attenuating the Antigen Presentation Ability of Monocyte Derived Dendritic Cells in Experimental Autoimmune Encephalomyelitis

Zhi-Yuan Shen[1†], Yi Zheng[2†], Maggie K. Pecsok[3], Ke Wang[4], Wei Li[1], Min-Jie Gong[5], Feng Wu[6] and Lin Zhang[1]*

[1] Department of Biochemistry and Molecular Biology, School of Basic Medicine, Xi'an Jiaotong University, Xi'an, China, [2] School of Medicine, University of Electronic Science and Technology of China, Chengdu, China, [3] Departments of Neurology and Immunology, School of Medicine, Yale University, New Haven, CT, United States, [4] MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou, China, [5] Department of Otolaryngology Head and Neck Surgery, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, [6] Center of Teaching and Experiment for Medical Post Graduates, School of Basic Medicine, Xi'an Jiaotong University, Xi'an, China

Experimental autoimmune encephalomyelitis (EAE) is a classical murine model for Multiple Sclerosis (MS), a human autoimmune disease characterized by Th1 and Th17 responses. Numerous studies have reported that C-reactive protein (CRP) mitigates EAE severity, but studies on the relevant pathologic mechanisms are insufficient. Our previous study found that CRP suppresses Th1 response directly by receptor binding on naïve T cells; however, we did not observe the effect on Th17 response at that time; thus it remains unclear whether CRP could regulate Th17 response. In this study, we verified the downregulation of Th17 response by a single-dose CRP injection in MOG-immunized EAE mice *in vivo* while the direct and indirect effects of CRP on Th17 response were differentiated by comparing its actions on isolated $CD4^+$ T cells and splenocytes *in vitro*, respectively. Moreover, the immune cell composition was examined in the blood and CNS (Central Nervous System), and a blood (monocytes) to CNS (dendritic cells) infiltration pathway is established in the course of EAE development. The infiltrated monocyte derived DCs (moDCs) were proved to be the only candidate antigen presenting cells to execute CRP's function. Conversely, the decrease of Th17 responses caused by CRP disappeared in the above *in vivo* and *in vitro* studies with $Fc\gamma R2B^{-/-}$ mice, indicating that FcγR2B expressed on moDCs mediates CRP function. Furthermore, peripheral blood monocytes were isolated and induced to establish moDCs, which were used to demonstrate that the antigen presenting ability of moDCs was attenuated by CRP through FcγR2B, and then NF-κB and ERK signaling pathways were manifested to be involved in this regulation.

OPEN ACCESS

Edited by:
Dipyaman Ganguly, Indian Institute of Chemical Biology (CSIR), India

Reviewed by:
Carolina Prado Terrazas, Fundación Ciencia and Vida, Chile
Tao Li, National Center of Biomedical Analysis (NCBA), China

***Correspondence:**
Lin Zhang
zhanglinxjtu@foxmail.com

[†]*These authors share first authorship*

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *04 August 2020*
Accepted: *01 March 2021*
Published: *25 March 2021*

Citation:
Shen Z-Y, Zheng Y, Pecsok MK, Wang K, Li W, Gong M-J, Wu F and Zhang L (2021) C-Reactive Protein Suppresses the Th17 Response Indirectly by Attenuating the Antigen Presentation Ability of Monocyte Derived Dendritic Cells in Experimental Autoimmune Encephalomyelitis. Front. Immunol. 12:589200. doi: 10.3389/fimmu.2021.589200

Ultimately, we perfected and enriched the mechanism studies of CRP in EAE remission, so we are more convinced that CRP plays a key role in protecting against EAE development, which may be a potential therapeutic target for the treatment of MS in human.

Keywords: experimental autoimmune encephalomyelitis, C-reactive protein, monocyte derived DC, FcγR2B, Th17 response

INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune disease in the human brain and spinal cord, referred to as central nervous system (CNS), that is characterized by immune cell infiltration, neuroinflammation, demyelination, and axonal damage (1, 2). Although the exact etiology of MS is unclear, it is generally considered to involve environmental, genetic, and immunological factors, and the immunopathology especially has been established and accepted for the last four decades (3–6). Experimental autoimmune encephalomyelitis (EAE) is a widely used murine model of MS, and a plethora of studies have shown that myelin specific $CD4^+$ T cells have a crucial role in the induction of EAE (7, 8).

CRP is an evolutionarily conserved pentamer plasma protein, and its plasma concentration increases dramatically as high as 1,000-fold during tissue injury and infection (9, 10). In the clinical setting, CRP is generally recognized as a non-specific inflammatory marker. As an innate immune molecule, CRP usually recognizes the Fc receptors to activate the classical pathway of complement and opsonize the phagocytosis of phagocytes in host defense (11, 12). Nevertheless, accumulating evidence indicates that CRP also plays an important role in some autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and EAE (13–15). Among these, EAE has been studied most extensively in recent years, and it is reported that CRP has a protective role in MOG/MBP immunized EAE with human CRP transgenic (*hCRP*tg) mice or single dose human CRP injection (13, 16). However, the anti-inflammatory mechanism of CRP in EAE is still unclear and needs to be further investigated. IFN-γ-producing helper T cells (Th1 cells) and IL-17-producing helper T cells (Th17 cells) are crucial mediators in both EAE and MS (17, 18). We previously found that CRP suppresses Th1 response by direct receptor binding to naïve T cells, and that Th1 response also was reduced in MOG-immunized EAE model after CRP treatment (16), whereas we did not detect changes in IL-17 or RORγt when $CD4^+$ T/naïve T cells were incubated directly with CRP at that time, so we wanted to investigate whether CRP regulates Th17 response in EAE pathogenesis.

The specific interactions between CRP and T cells are poorly studied, with initial reports saying that CRP could bind with T cells and thereby mediate the effective function in mid-1970s, but later this binding was denied by the same group (19, 20). Although we proved the direct binding of naïve T cells and pentamer CRP, this binding was not associated with Th17 regulation. Therefore, we moved our attention to antigen presenting cells (APCs), which usually express CRP's three traditional receptors (21, 22). APCs internalize extracellular antigens and present MHC-I/II binding antigen fragments to T cells, generating antigen-specific $CD4^+$ T cells (23). Some existing studies reported that CRP participates in $CD4^+$ T cell responses through APCs, including monocytes, dendritic cells (DCs), and macrophages (24–26). However, we still don't know which type of APC mediates the CRP's function in EAE. Even though the Szalai group reported recently that CRP impairs DC maturation and function, thereby affecting $CD4^+$ T cell responses in EAE development (27), it is still unclear why they focused on DCs directly and where did these DCs come from during EAE onset. Moreover, it is only a theoretical possibility that CRP affects T cell responses by inhibiting APC maturation, as only T cell proliferation was assessed in previous studies, and the $CD4^+$ effector T cells have never been detected so far (24, 27). In this paper, we focus on the effects of CRP on Th17 response, and Th1, Th2, and Treg are assessed for the first time. Furthermore, we will investigate the moDCs' antigen presentation ability regulated by CRP, something which remains controversial (24, 27, 28), as well as the signaling pathways involved in altering the antigen presenting molecules by CRP.

With this paper, we demonstrated that CRP regulates Th17 response indirectly by influencing the antigen presenting ability of moDCs though FcγR2B. In addition, the mechanisms by which CRP inhibits EAE development are further completed and elaborated. These findings not only provide profound insight into the contribution of CRP in host defense, but also put forward new ideas and potential targets for the intervention of autoimmune diseases.

MATERIALS AND METHODS

Reagents

Human pentamer CRP, purified from ascites (purity>99%), was purchased from the Binding Site (BP300.X, Birmingham, United Kingdom). Generally, CRP was treated to further purification with immobilized p-Aminophenyl Phosphoryl Choline (Cat: 20307, Lot: RH237939,Thermo Fisher Scientific, Waltham, MA, USA) and a Detoxi-Gel column (20344, Thermo Fisher Scientific) packed with Polymixin B ligand immobilized on beaded affinity resin to bind and extract endotoxins from protein samples, as has been described in our previous studied (29, 30). Antibodies to p-STAT1 (Cat: 9167s, Lot: 4), STAT1 (Cat: 9172s, Lot: 25), STAT3 (Cat: 4904s, Lot: 7), p-STAT3 (Cat: 9145s, Lot: 34), p-ERK1/2 (4370s, Cat: 2), ERK1/2 (Cat: 4695s, Lot: 1) and NF-κB p65 (Cat: 8242s, Lot: 1) were purchased from

Cell Signaling Technology (Danvers, MA, USA). Anti-mouse CD25 APC (Cat: 17-02510-82, Lot: 4276862), anti-mouse MHC-II APC(I-A/I-E) (Cat: 17-5321-81, Lot: 1991457), anti-mouse CD86 APC (Cat: 17-0862-81, Lot: 1984132), anti-CD3 mAb (Cat: 16-0031-85, Lot: 4349473), anti-CD28 mAb (Cat: 16-0281-85, Lot: 1974623), antibodies to RORγt (Cat: 14-6981-82, Lot: 1936480), T-bet (Cat: 14-5825-82, Lot: 2012147) and Mouse IL-17AF ELISA Set (Cat: 88-8711-88, Lot: 4291151) were obtained from ebioscience (San Diego, CA, USA). Anti-mouse CD4 FITC (Cat: 553046, Lot: 5027567), anti-mouse IFN-γ PerCP-Cy5.5 (Cat: 560660, Lot: 5244738), anti-mouse IL-17A PE (Cat: 559502, Lot: 8071502), anti-mouse CD11b PE (Cat: 557397, Lot: 9023691), anti-Mouse CD45 APC (Cat: 559864, Lot: 8277680), anti-Mouse CD11c FITC (Cat: 557400, Lot: 8060996), anti-Mouse CD45R/B220 FITC (Cat: 553087, Lot: 8152878), Mouse IFN-γ ELISA Set (Cat: 555138, Lot: 7192700), Mouse IL-10 ELISA Set (Cat: 555252, Lot: 6154834), BD Pharm lyse™ (Cat: 555899, Lot: 8250695), Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (Cat: 555028, Lot: 5261614) were purchased from BD Biosciences (San Jose, CA, USA). Anti-mouse PD-L1 APC (Cat: 124311, Lot: B277024) and anti-mouse OX40L APC (Cat: 108811, Lot: B274358) were purchased from Biolegend (San Diego, CA, USA).

Animals

Wild-type mice (strain C57BL/6) were from the Experimental Animal Center of Xi'an Jiaotong University. $CRP^{-/-}$mice were generated through Shanghai Model Organisms Co. Ltd (Shanghai, China). $Fc\gamma R2B^{-/-}$ mice were purchased from Jackson Lab (Bar Harbor, Maine, USA). All mice were housed in the same vivarium at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12-h light/dark cycle. All procedures for the use of animals were approved by the Animal Ethics Committee of Xi'an Jiaotong University.

Induction and Evaluation of EAE

Experimental autoimmune encephalomyelitis (EAE) was induced as we described previously (16). Briefly, 10–12 week old female mice were immunized subcutaneously with 200 μg myelin oligodendrocyte glycoprotein (MOG, MEVGWYRSP FSRVVHLYRNGK) peptide 35–55 (≥99% purity, Shanghai Science Peptide Biological Technology, Shanghai, China) in complete Freund's adjuvant containing 4 mg *Mycobacterium tuberculosis* strain H37Ra (Cat: 7027, Lot: 180226, Chondrex, Redmond, WA, USA). On days 0 and 2, immunized mice received an intraperitoneal injection of 200 ng pertussis toxin (PTX, Cat: 181, Lot: 181238A1, List Biological Labs, CA, USA). On day 2, immunized mice received a single intraperitoneal injection of 200 μg human CRP or control buffer, and then the development of EAE was monitored daily. Neurological impairment was quantified daily on an arbitrary clinical scale: 0, asymptomatic; 1, decrease of tail tonicity; 2, limp tail and weakness of hind limb; 3, limp tail and partial hind limb paralysis; 4, limp tail, complete hind limb and partial foreleg paralysis; 5, moribund (31, 32). The splenocytes were isolated at the peak of EAE symptoms and re-stimulated *in vitro* with 50 μg/ml MOG peptide 35–55. Flow cytometry and ELISA determined intracellular cytokines and secreted cytokines respectively.

Splenocytes and $CD4^+$ T Cells' Separation

Splenocytes were directly obtained from the spleens after removing the red cells by BD Pharm lyse™. $CD4^+$ T cells were purified from the spleens using MACS kits (Cat: 130-049-201, Miltenyi Biotec, Bergisch Gladbach, Germany). The splenocytes and $CD4^+$ T cells were cultured in RPMI 1640 medium (Cat: 11875-093, Gibco) containing 10% fetal bovine serum (BISH5400, BI), 1% penicillin/streptomycin, 50 uM 2-mercaptoethanol and were maintained in a humidified incubator with 5% CO2 at 37°C overnight. The cells were treated in 96-well culture plates (2.5×10^5 cells in 300 ul per well) with plate-bound anti-CD3 (2 μg/ml, immobilized overnight at 4°C) and fluid phase anti-CD28 (2 μg/ml), in the presence or absence of CRP (100 μg/ml), and then collected after 24 h for mRNA detection and 72 h for protein detection.

Th Cell Differentiation

The splenocytes and $CD4^+$ T cells were obtained and cultured 3 days under Th1-polarizing conditions (10 ng/ml mIL-2, 20 ng/ml mIL-12p70, and 10 mg/ml anti-IL-4 mAb) or Th17-polarizing conditions (25 ng/ml IL-6, 5 ng/ml TGF-β, 20 ng/ml IL-1β, 10 μg/ml anti-IL-4 mAb and 10 μg/ml anti-IFN-γ mAb) (33, 34). The cells were transferred to a 24-well plate for an additional 2 days' expansion. At the end of the culture, PMA (20 ng/ml), ionomycin (1 mg/ml), and BD GolgiPlug protein transport inhibitor containing brefeldin A were added for 4 h incubation. Cells were then collected for Flow Cytometry analysis.

Immune Cells' Isolation From Peripheral Blood and CNS

Mice were anesthetized by tribromoethanol, and the blood was collected from the eyeballs of mice. Mice were then perfused through the left ventricle with cold PBS. Brains and spinal cords were dissected and grounded through a cell strainer (70 μm), then re-suspended in 3 ml 30% percoll (GE, Cat: 17-0891-01, Lot: 1024671), and layered slowly on top of the 10 ml 70% percoll. After 30 min centrifugation at 800×g at 18°C (Acceleration,3; Deceleration,2), the layer of debris from the top of the tube was gently removed. Mononuclear cells were collected from the 70 to 30% interphase into a clean conical tube with 8 ml of 1× HBSS and washed for three times by centrifugation for 10 min at 500×g at 18°C. Finally, the cells were re-suspended in culture medium for flow cytometry.

moDCs' Generation, Culture, and Activation

Mouse moDCs were isolated from peripheral blood mononuclear cells (PBMCs) of 6–8 weeks old mice. Briefly, PBMCs were obtained through Ficoll gradient centrifugation, and after a 2 h adherence in 75 cm^2 flasks, the non-adherent cells were removed and the adherent cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 20 ng/ml GM-CSF (Cat: 415, Lot: BJ2519024, R&D), 10 ng/ml IL-4 (Cat: 550067, Lot:

8151542, BD Biosciences). The culture medium was replaced 3/4 on days 2 and 4. On day 6, the cells were harvested and seeded (1×10^5/ml) in 24-well plates with or without CRP (100 μg/ml), and LPS (1 ug/ml) was added for moDC maturation on day 8. The moDCs were then harvested for flow cytometry and western blot on day 9.

Real-Time PCR and Western Blot

Total RNA was extracted with RNAiso Plus reagent (9190, Takara, Shiga, Japan), and reverse transcribed using a Prime Script RT Master Mix Kit (RR036A, Takara). The target genes were quantified by quantitative real-time PCR using RealStar Green Power Mixture (A311, Genestar, Beijing, China) in a StepOne Plus real-time PCR system (Thermo Fisher Scientific).The primer sequences used were: GAPDH (forward: 5′-GGAGAAACCTGCCAAGTATGA-3′; reverse: 5′-GTGGGTGCAGCGAACTTTA-3′); IL-17 (forward: 5′-GCTGACCCCTAAGAAACCCC-3′; reverse: 5′-GTCCACAGAAAAACAAACACGA-3′); IFN-γ (forward: 5′-CGGCACAGTCATTGAAAGCCTA-3′; reverse: 5′-CTCTGCAGGATTTTCATGTCACC-3′); IL-4 (forward: 5′-TTCCAAGGTGCTTCGCATA-3′; reverse: 5′-TGCAGCTTATCGATGAATCCA-3′); IL-10 (forward: 5′-GCCTTATCGGAAATGATCCAGT-3′; reverse: 5′-GAAATCGATGACAGCGCCTC-3′); RORγt (forward: 5′-GGATGAGATTGCCCTCTACAC-3′; reverse: 5′-AGGAGGCCTTGTCGATGAG-3′); T-bet (forward: 5′-CCATTCCTGTCCTTCACCG-3′; reverse: 5′-CTGCCTTCTGCCTTTCCAC-3′); GATA-3 (forward: 5′-CTGGATGGCGGCAAAGC-3′; reverse: 5′-GTGGGCGGGAAGGTGAA-3′); Foxp3 (forward: 5′-AAGTACCACAATATGCGACCC-3′; reverse: 5′-GTAGGCGAACATGCGAGTAA-3′); MHCII (forward: 5′-TTACCAAGTACGGCAACATGACC-3′; reverse: 5′-AGATCTTCCAGTTCACGCCAT-3′); CD86 (forward: 5′-ACGCAAGCTTATTTCAATGGGA-3′; reverse: 5′-AAATAGTGCTCGTACAGAACCA-3′); CD80 (forward: 5′-TTGCCGTTACAACTCTCC-3′; reverse: 5′-GTTCTTATACTCGGGCCACA-3′); CD70 (forward: 5′-CGCCTGACATACCTGGTCCAC-3′; reverse: 5′-AGGGCATATCCACTGAACTCC-3′); ICOS-L (forward: 5′-ACACAACGGACAATAGCCTA-3′; reverse: 5′-GGAGAGCCACATTCTCTACGC-3′); PD-L1 (forward: 5′-GTCAATGCCCCATACCGCAAA-3′; reverse: 5′-TTCTCTTCCCACTCACGGGTT-3′); PD-L2 (forward: 5′-GCCTCTACCAGGTCACCAGT-3′; reverse: 5′-ACTTTGGGTTCCATCCGACT-3′); Ox40L (forward: 5′-ATTGACCTTCATTTCCGGGAG-3′; reverse: 5′-AGTATCAGGAGCATTTACAGT-3′); BTLA (forward: 5′-CCCCTTGAAGTTGGTCCTC-3′; reverse: 5′-TGTAGAACAGCTATACGACCC-3′); HVEM (forward: 5′-ATTCCTCATCTGCACGCGAAG-3′; reverse: 5′-CAGCAAACCCAACCTCGGTGA-3′); SLAM (forward: 5′-TCCCCTCCAGAGATTAAAGTGC-3′; reverse: 5′-TGTAAGTCACATGGTCCCCTT-3′); 4-1BBL (forward: 5′-AACAAGTTAGTGGACCGTTCCT-3′; reverse: 5′-GCTCCATGCAGATAAGCCCTCA-3′).

Cells were lysed in RIPA lysis buffer (10 mM Tris-HCL at pH 9.6, 1 mM EDTA, 150 mM NaCl, 1% NP-40, and 0.5 mM PMSF) supplemented with protease/phosphatase inhibitor. Proteins were denatured and electrophoresed in 8% Glycine-Tris/polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked by TBST containing 5% BSA for 2 h and incubated in primary antibodies at 4°C overnight, labeled horseradish peroxidase conjugated antibodies and detected using a Fusion FX System. The blots were analyzed and quantified by Image J software.

Histological Staining and Analyzing

After mice were sacrificed, the spinal cords were fixed with 10% neutral formalin, dehydrated and embedded in paraffin, and then cut into 5-mm sections. After drying at 42°C overnight, the spinal sections were dewaxed in xylol, rehydrated, stained with hematoxylin–eosin (H&E) and luxol fast blue (LFB), according to the manufacturers' instructions. Tissue inflammation and demyelination were assessed by Image J pro software.

Flow Cytometry

Spleen single cell suspension and cultured differentiated T cells were prepared after PBS washing; anti-mouse CD16/32 mAb (AF1460, Abcam, MA, USA) was used to block the non-specific binding. The combination of surface staining and intra-cellular cytokines staining was used in FACS. For surface makers' staining, cells and fluorescent antibodies (for CD3, CD220, CD45, CD4, CD25) were incubated directly for 30 min at 4°C. Cells were fixed and permeabilized with a Fixation/Permeabilization Solution Kit (555028, BD Biosciences) for 20 min at room temperature before cytokine staining. For intracellular staining, specific fluorescent antibodies for cytokines (IFN-γ, IL-17, IL-4, IL-10) were incubated with cells for 30 min at 4°C. All FACS antibodies for flow cytometry were purchased from BD Biosciences and used according to the manufacturer's instructions. Analysis was performed using a Beckman Coulter Cytoflex Flow Cytometer and FlowJo software.

Statistical Analysis

Data are presented as the means ± SEM. Statistical analysis among groups was performed using one-way ANOVA with Tukey *post hoc* test. All statistical analyses were performed using Graph Pad Prism 7.0; $p < 0.05$ was considered statistically significant.

RESULTS

Th17 Responses Are Inhibited in Wild Type EAE Mice With One-Dose CRP Injection

To investigate whether CRP participates in Th17 response in EAE, WT C57BL/6 mice were immunized by myelin oligodendrocyte glycoprotein (MOG) in complete Freund's adjuvantand pertussis toxin, with a single-dose injection of human CRP treatment. Unsurprisingly, the CRP-treated EAE mice showed a milder state of pathology than the vehicle-treated EAE mice (TBS-Ca^{2+}), assessed by clinical score, body weight, HE, and LFB staining (**Supplementary Figure 1**), which is in agreement with published data (13, 16). We next collected the splenocytes to re-stimulate with MOG to evaluate the activation

of $CD4^+$ T cell subsets at the peak of EAE disease. The results showed that IL-17 and RORγt expression were high in EAE vehicle mice, while they were significantly decreased in CRP-treated EAE mice (**Figure 1A**). The IFN-γ and T-bet expression were also greatly reduced in CRP-treated EAE mice compared to their untreated littermates (**Figure 1B**), which is consistent with our previous findings (16). Meanwhile, there was no significant difference in the expression of IL-4 or GATA-3, genes relevant for Th2 function (**Figure 1C**); or in the expression of IL-10 or Foxp3, genes relevant for Treg function (**Figure 1D**). These data were further validated at a single cell level by flow cytometry analysis of Th17 and Th1 cells. As shown in **Figure1E**, $CD4^+$ $IL\text{-}17^+$ T cells and $CD4^+$ IFN-γ^+ T cells were lower in CRP-treated EAE mice than in vehicle-treated EAE mice ($CD4^+$ T cells were gated first), whereas Th2 and Treg subsets did not exhibit these changes and were always low in abundance (data not shown). These data indicated that Th17 responses were suppressed in MOG-induced EAE by CRP injection.

CRP Suppresses the Th17 Responses Only in Splenocytes, But Not Isolated $CD4^+$ T Cells

As we discussed in the *Introduction*, we hypothesized that CRP could participate in Th17 response indirectly in virtue of antigen presenting cells to shape T cell responses in EAE. However, it was too premature to say definitively which kind of APCs mediates this process. To verify our hypothesis and simulate the direct and indirect effects of CRP on Th17 response, we employed magnetic beads isolated $CD4^+$ T cells and erythrocyte-lysed splenocytes, respectively, from WT mice to incubate with human CRP and vehicle. It should be emphasized that splenocytes contain not only all the kinds of APCs but also T cells; yet, since there was no

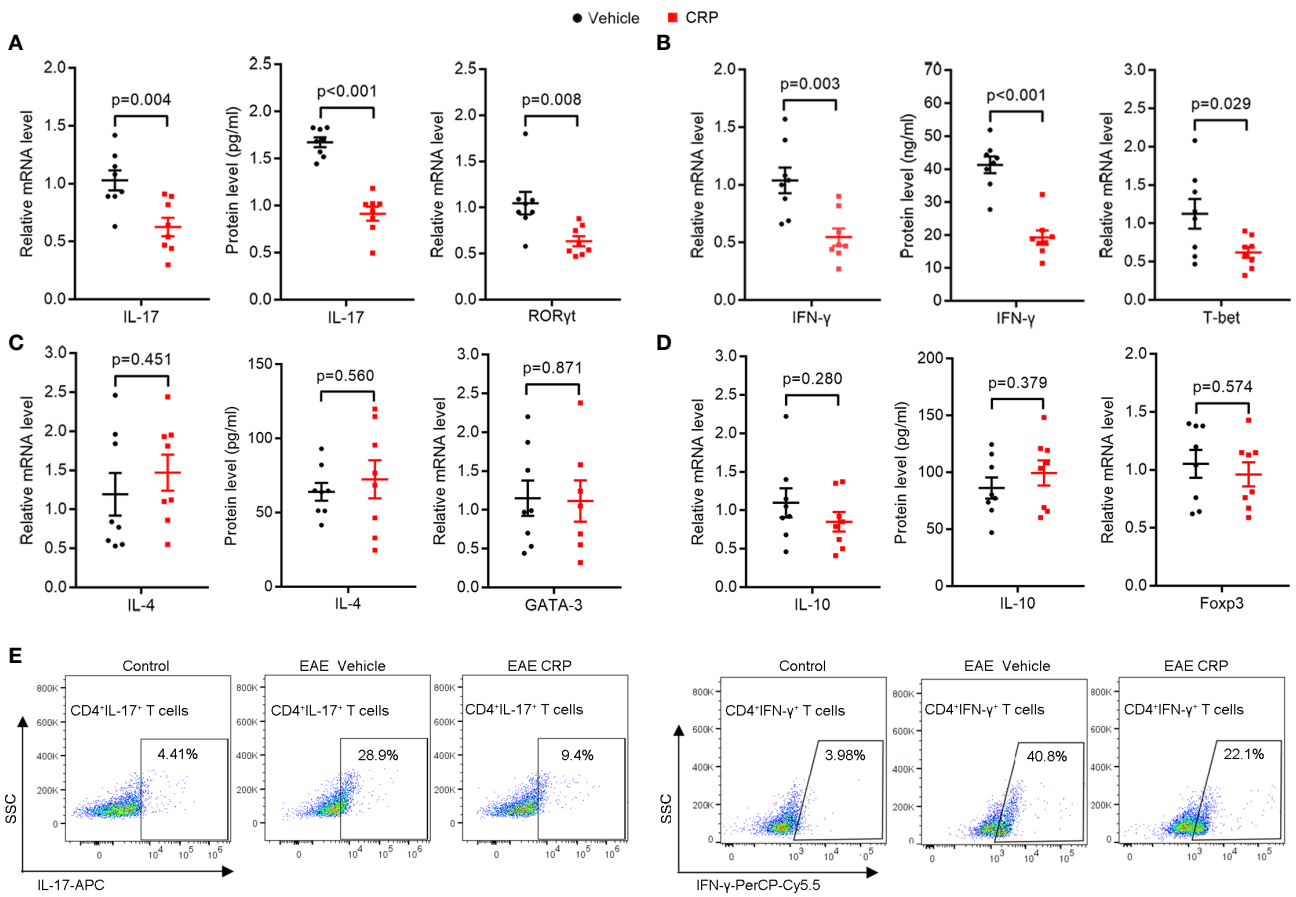


FIGURE 1 | The Th17 responses are suppressed by one-dose CRP injection in WT EAE mice.Splenocytes were isolated form EAE and EAE CRP mice, then re-stimulated with 50 ug/ml MOG 24 h for qPCR, 72 h for ELISA and FACS. **(A)** Th17 relevant cytokine (IL-17) and transcription factor (RORγt) were examined by qPCR and ELISA (n = 8). **(B)** Th1 relevant cytokine (IFN-γ) and transcription factor (T-bet) were measured by qPCR and ELISA (n = 8). **(C)** Th2 relevant cytokine (IL-4) and transcription factor (GATA-3) were detected by qPCR and ELISA (n = 8). **(D)** Th2 relevant cytokine (IL-10) and transcription factor (Foxp3) were tested by qPCR and ELISA (n = 8). **(E)** Th17 ($CD4^+$ $IL\text{-}17^+$) and Th1 ($CD4^+$ IFN-γ^+) cells were examined by FACS (n = 3). Data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant.

direct effect of CRP on Th17 cells, the splenocytes served to model an indirect APC-mediated regulation of CRP on Th17. We first measured the expression of IL-17 after direct incubation of CRP with splenocytes and $CD4^+$ T cells. These results showed that the level of IL-17 in the splenocytes group was obviously lower in CRP-treated samples than in vehicle samples, while in $CD4^+$ T cell group, there was no change with CRP treatment. This finding was consistent in protein and gene levels (**Figures 2A, B**). At the same time, the expression of IFN-$\gamma$was weakened with CRP treatment in both splenocytes and $CD4^+$ T cells (**Figures 2A, B**). To determine whether CRP affects the Th17/Th1 differentiation, we cultured the splenocytes and $CD4^+$ T cells in Th17 and Th1 differentiation conditions to examine the expression of transcription factors and signal transducer and activator of transcription (STATs) by western blotting. Here, TGF-β, IL-1β and IL-6 were used to induce Th17 differentiation, while IL-12 was used to induce Th1 differentiation, then RORγt and p-STAT-3 were examined in Th17 differentiation, while T-bet and p-STAT-1 were examined in Th1 differentiation. RORγt and p-STAT-3 were down-regulated with CRP stimulation in the splenocytes, but not in $CD4^+$ T cells (**Figures 2C, D**). On the contrary, T-bet and p-STAT-1 expressions were substantially diminished in CRP-treated splenocytes and $CD4^+$ T cells compared to vehicle (**Figures 2C, D**). Collectively, our data clearly demonstrated that the Th17 response was attenuated indirectly by CRP treatment *in vitro* experiments, that is, with the help of APCs, but Th1 response could be regulated both directly and indirectly by CRP.

Monocyte Derived $CD11b^+$ $CD11c^+$ DC Cells Mediate the Interaction of CRP on Th17 Response

Immune cell infiltration is one of the core events in EAE development (35, 36). In addition to the encephalitogenic T cells, antigen presenting cells including B cells, macrophages, and DCs can infiltrate into the CNS and theoretically participate in T cell responses. Moreover, published reports have proposed that CRP influences the expression of chemokines and chemokine receptors, and thus participates in the migration and movement of immune cells into the CNS to modulate neuroinflammation (37, 38). To identify the candidate APCs that mediate the Th17 response by CRP and to see whether CRP affects the degree of

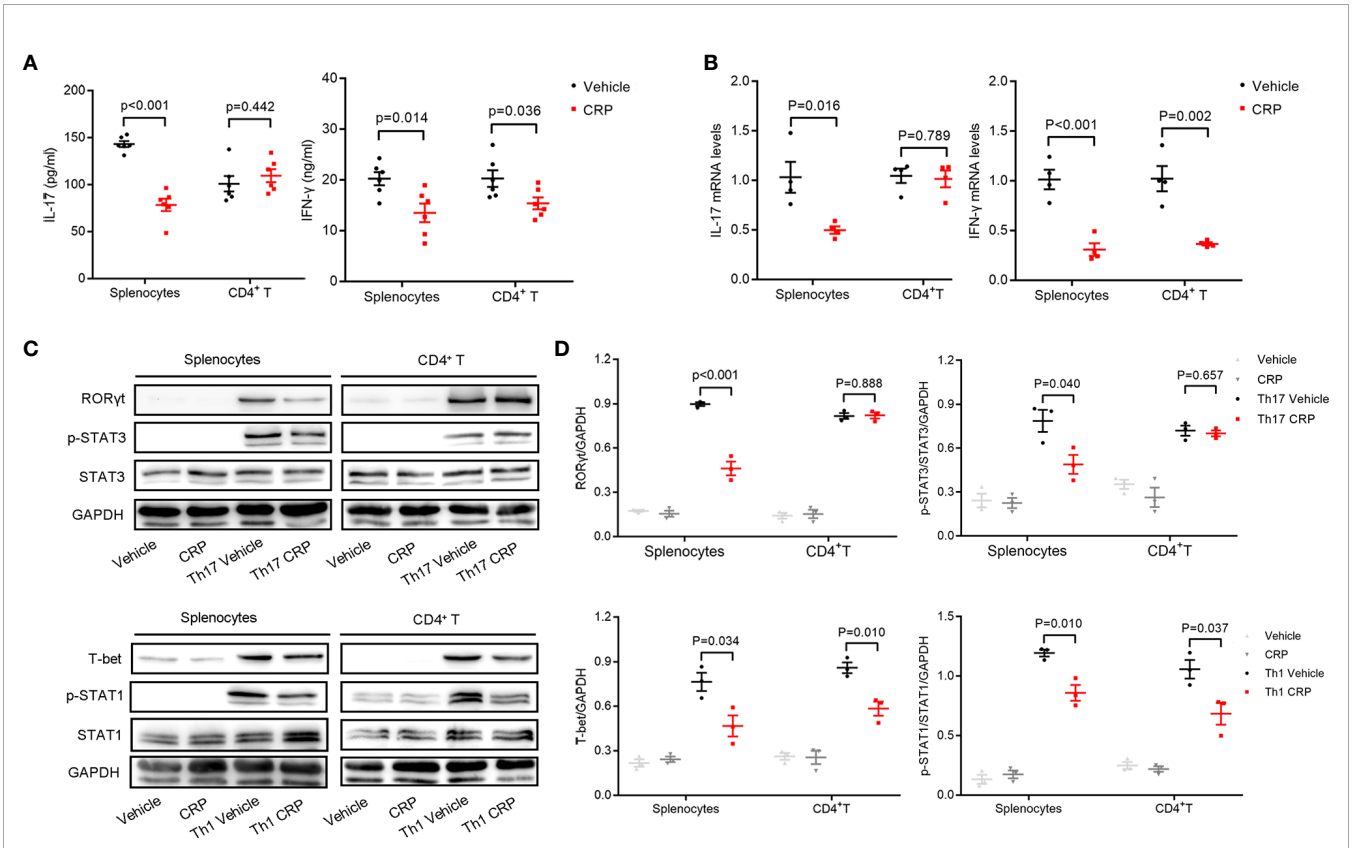


FIGURE 2 | CRP reduces Th17 responses in splenocytes from WT mice.Splenocytes and $CD4^+$ T cells were isolated from WT mice to distinguish the indirect and direct regulations of CRP on Th17. **(A)** The protein expression levels of IL-17 and IFN-γwere measured with CRP treatment in both splenocytes and $CD4^+$ T cells by ELISA (n = 6). **(B)** The mRNA expression levels of IL-17 and IFN-γwere measured with CRP treatment in both splenocytes and $CD4^+$ T cells by qPCR (n = 4). **(C)** Splenocytes and $CD4^+$ T cells were cultured with CRP in Th17 and Th1 polarization conditions, then RORγt, T-bet, p-STAT-3, and p-STAT-1 were examined by WB. **(D)** The quantitative and statistical analysis of the WB results was presented (n = 3). Data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant.

immune cell infiltration to trigger Th17 response, the immune cell composition of blood and CNS from healthy mice (control), EAE mice and CRP-treated EAE mice were analyzed by flow cytometry. Our data revealed that compared to control mice, EAE mice maintained a similar percentage of T cells ($CD45^+CD3^+$ T cells) in the blood, whereas in CNS, T cells were increased from 5 to 25% of total immune cells in EAE mice (**Figures 3A, E, F**). The percentage of B cells ($CD45^+B220^+$ B cells) was decreased from 55 to 25% in the blood, but they had no obvious difference in the CNS between control and EAE mice (**Figures 3B, E, F**). Unexpectedly, the percentage of blood monocytes was raised from 30 to 80% in EAE mice compared

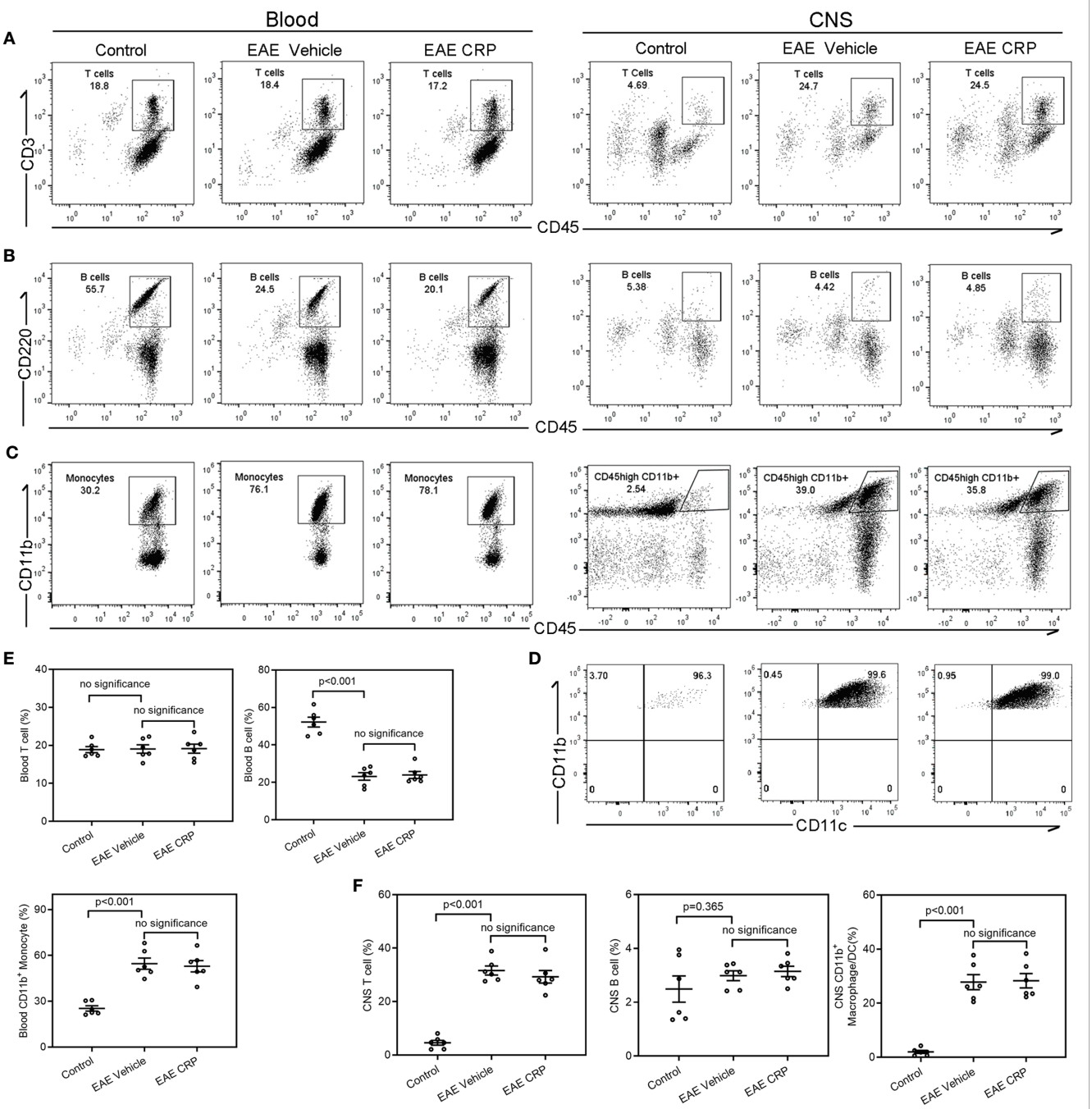


FIGURE 3 | Analysis of immune cell composition of blood and CNS during the effector phase of EAE in WT mice.Flow cytometry of T cells **(A)**, B cells **(B)** and monocytes **(C)** in the blood and CNS from WT control mice, WT EAE vehicle mice and WT EAE CRP mice (n = 6). **(D)** The CNS-infiltrating $CD45^{high}$ $CD11b^+$ myeloid cells were proved to be $CD11b^+$ $CD11c^+$ DCs. **(E)** The quantitative and statistical analyses of the immune cells in blood were presented (n = 6). **(F)** The quantitative and statistical analyses of the immune cells in CNS were presented (n = 6). Data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant. ns, no significance.

to control mice, and in $CD45^+CD11b^+$ cells its level was elevated from 3 to 40% in the CNS during EAE (**Figures 3C, E, F**). Further analysis showed that the population of CD45high $CD11b^+$ cells were $CD11b^+CD11c^+$ DCs (**Figure 3D**). In addition to T cells, the majority of immune cells that infiltrated into the CNS were $CD11b^+$ $CD11c^+$ DCs in EAE mice. Notably, CRP treatment did not affect the ratio of T cells, B cells, and monocytes/macrophages/DCs in EAE mice. Therefore, it was plausible that moDCs may be the mediators of CRP-triggered Th17 response and that CRP did not alter the percentages of these immune cells in the blood and CNS.

FcγR2B on moDCs Mediates the Regulation of CRP on Th17 Response

There are three major CRP receptors expressed on DCs, including FcγRI, FcγRII, and FcγRIII (39–41), but the FcγR2B is the only one that has been reported to mediate CRP function in EAE (27, 42). We were therefore interested in whether FcγR2B on moDCs could mediate the CRP function on Th17. We constructed the same EAE model using $Fc\gamma R2B^{-/-}$ mice as we performed in WT mice and found that the pathology of $Fc\gamma R2B^{-/-}$ EAE mice was slightly ameliorated with CRP injection as evidenced by clinical score, weight, and histochemistry analysis (**Supplementary Figure 1**), suggesting that CRP can regulate EAE not only by suppressing the response of Th1 cells, but also by an APCs independent mechanism. We next isolated the splenocytes for MOG re-stimulation from $Fc\gamma R2B^{-/-}$ EAE mice with and without CRP treatment and found that the levels of IL-17 and RORγt expression were unaltered between these two groups (**Figure 4A**), which was opposed to the results obtained from WT mice (**Figure 1A**). However, IFN-γ and T-bet expression remained decreased in

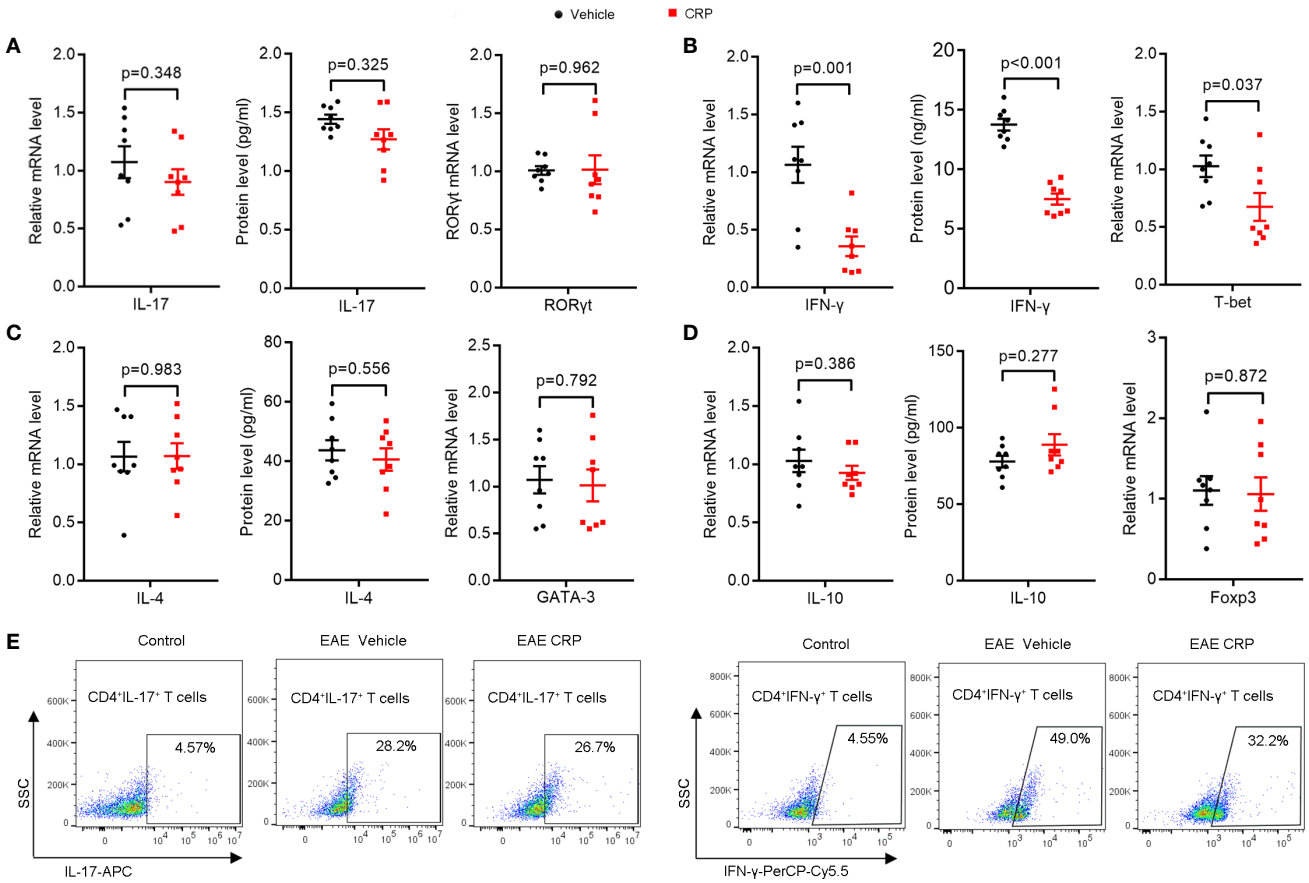


FIGURE 4 | The decrease in Th17 response disappeared in $Fc\gamma R2B^{-/-}$ EAE mice with CRP injection. **(A)** qPCR and ELISA analysis of IL-17 and RORγt expression in splenocytes and $CD4^+$ T cells from $Fc\gamma R2B^{-/-}$ EAE mice and $Fc\gamma R2B^{-/-}$ EAE CRP (n = 8). **(B)** qPCR and ELISA analysis of IFN-γ and T-bet expression in splenocytes and $CD4^+$ T cells from $Fc\gamma R2B^{-/-}$ EAE mice and $Fc\gamma R2B^{-/-}$ EAE CRP (n = 8). **(C)** qPCR and ELISA analysis of IL-4 and GATA-3 expression in splenocytes and $CD4^+$ T cells from $Fc\gamma R2B^{-/-}$ EAE mice and $Fc\gamma R2B^{-/-}$ EAE CRP (n = 8). **(D)** qPCR and ELISA analysis of IL-10 and Foxp3 expression in splenocytes and $CD4^+$ T cells from $Fc\gamma R2B^{-/-}$ EAE mice and $Fc\gamma R2B^{-/-}$ EAE CRP (n = 8). **(E)** Flow cytometry analysis of Th17 ($CD4^+$ $IL\text{-}17^+$) and Th1 ($CD4^+$ $IFN\text{-}\gamma^+$) cells by cell surface and intercellular staining from $Fc\gamma R2B^{-/-}$ EAE mice and $Fc\gamma R2B^{-/-}$ EAE CRP (n = 3). Data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant.

these two groups, which were consistent with WT mice (**Figures 4B** and **2B**). Also, there were no expression difference of IL-4, GATA-3, IL-10, and Foxp3 by MOG re-stimulation in the above two groups (**Figures 4C, D**). In addition, the decrease of $CD4^+$ $IL\text{-}17^+$ T cells in WT mice disappeared in $Fc\gamma R2B^{-/-}$ mice, but the $CD4^+$ $IFN\text{-}\gamma^+$ T cells' percentage was still reduced in $Fc\gamma R2B^{-/-}$ mice as in WT mice (**Figure 4E**), $CD4^+$ $IL\text{-}4^+$ T cells, and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells had not detected the signals (data not shown).

Furthermore, splenocytes and $CD4^+$ T cells were isolated from $Fc\gamma R2B^{-/-}$ mice and incubated with or without CRP to look at whether FcγR2B mediates the CRP function on Th17 *in vitro* experiments. The ELISA analysis revealed that the IL-17 expression had no significant changes in splenocytes or $CD4^+$ T cells with CRP stimulation from $Fc\gamma R2B^{-/-}$ mice, while the IFN-γ expression was diminished in both splenocytes and $CD4^+$ T cells in $Fc\gamma R2B^{-/-}$ mice (**Figure 5A**). Further, qPCR analysis yielded similar results (**Figure 5B**). Moreover, we assessed the RORγt/T-bet and p-STAT-3/p-STAT-1 expressions under Th1 and Th17 polarization conditions in $Fc\gamma R2B^{-/-}$ mice by WB. These data showed that RORγt and p-STAT-3 expression were unaltered with CRP treatment both in splenocytes and CD4+ T cells under Th17 polarization, whereas under Th1 polarization, the expressions of T-bet and p-STAT-1 remained down-regulated by CRP treatment both in splenocytes and $CD4^+$ T cells (**Figure 5C**). Statistical and quantitative analysis of WB were shown in **Figure 5D**. Collectively, our data clearly demonstrated that FcγR2B mediated the function of CRP on Th17 response *in vivo* and *in vitro*.

CRP Attenuates the Capability of Antigen Presentation of CNS Infiltrated moDCs Through FcγR2B

We have proven that the increased monocytes in the blood infiltrate the CNS and differentiate into DCs during EAE development. Meanwhile, because we did not detect any percentage changes of these infiltrated immune cells caused by CRP treatment, so we speculated that CRP may reduce the antigen presentation ability of these monocyte derived DCs (moDCs) to participate in Th17 response and which could be

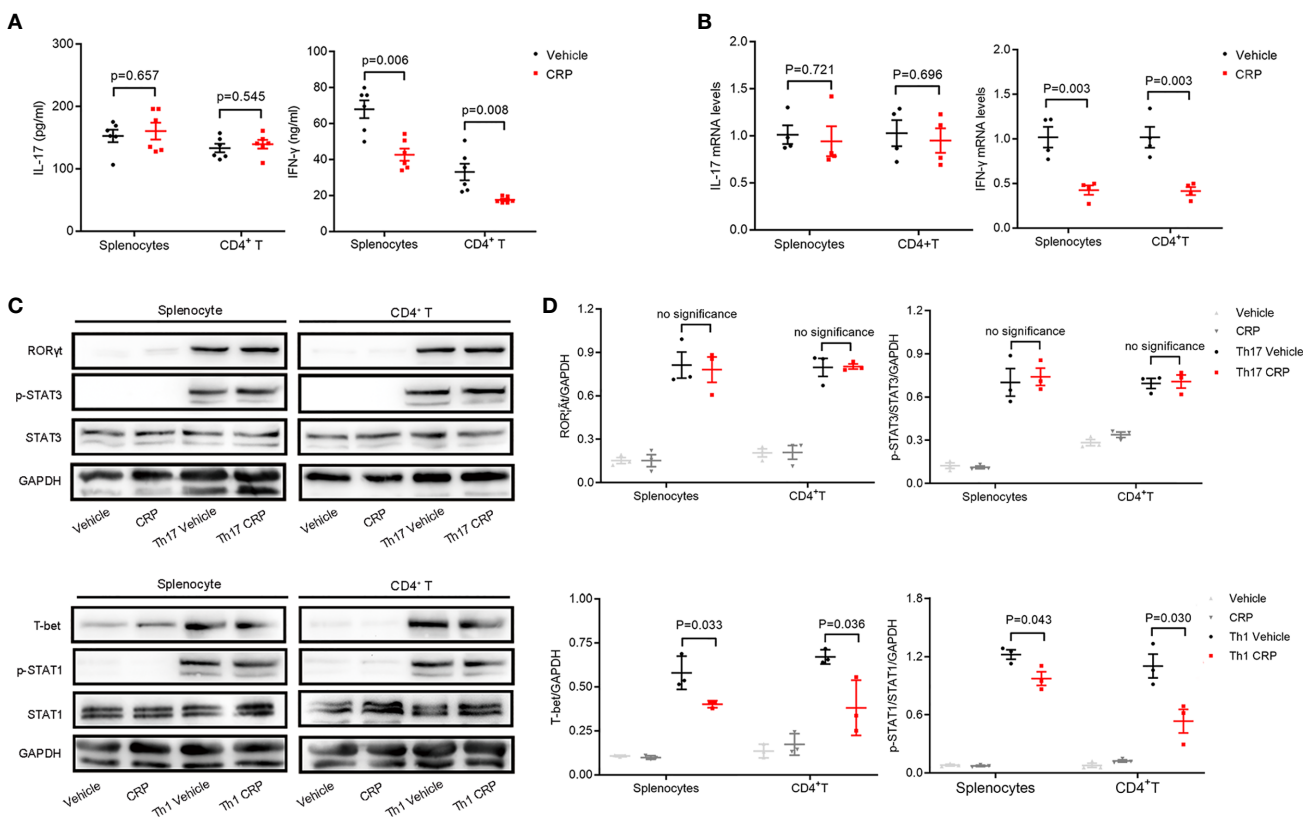


FIGURE 5 | The decrease in Th17 response disappeared in $Fc\gamma R2B^{-/-}$ splenocytes.Splenocytes and $CD4^+$ T cells were isolated from $Fc\gamma R2B^{-/-}$ mice to distinguish the indirect and direct regulations of CRP on Th17. **(A)** The protein expression levels of IL-17 and IFN-γ were measured in $Fc\gamma R2B^{-/-}$ splenocytes and $Fc\gamma R2B^{-/-}$ $CD4^+$ T cells with or without CRP treatment (n = 6). **(B)** The mRNA expression levels of IL-17 and IFN-γ in $Fc\gamma R2B^{-/-}$ splenocytes and $Fc\gamma R2B^{-/-}$ $CD4^+$ T cells with or without CRP treatment (n =4). **(C)** $Fc\gamma R2B^{-/-}$ splenocytes and $Fc\gamma R2B^{-/-}$ $CD4^+$ T cells were isolated and incubated with or without CRP in Th17 and Th1 polarization condition, then RORγt, T-bet, p-STAT-3, and p-STAT-1 were detected by WB (n = 3). **(D)** The quantitative and statistical analysis of the WB results was presented (n = 3). Data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant. ns, no significance.

mediated by FcγR2B expressed on moDCs. To test our speculation, moDCs were successfully isolated from PBMCs and established by GM-CSF and IL-4 as reported (43, 44), and LPS was added as a positive activator for antigen presentation molecules. Some crucial antigen presentation molecules were screened and verified in moDCs from WT mice by qPCR, including *MHC-II, CD86, CD80, CD70, COSL-1, PD-L1, PD-L2, OX40L, BTLA, HEVM, SLAM*, and *4-1BBL* (**Supplementary Figure 2A**), which were reported to be involved in modulating antigen presentation ability (45–47). The qPCR analysis showed that *MHC-II, CD86, PD-L1*, and *OX40L* had a lower expression in LPS CRP stimulation than LPS stimulation alone (**Figure 6A**). However, the difference in PD-L1 and OX40L expression was lost when we reexamined them by FACS (**Supplementary Figures 2B, C**). moDCs were next incubated with LPS from $CRP^{-/-}$ mice and WT mice, and we found that the expression of MHC-II and CD86 was increased in $CRP^{-/-}$ mice compared to moDCs with LPS from WT mice. Even with no LPS, moDCs

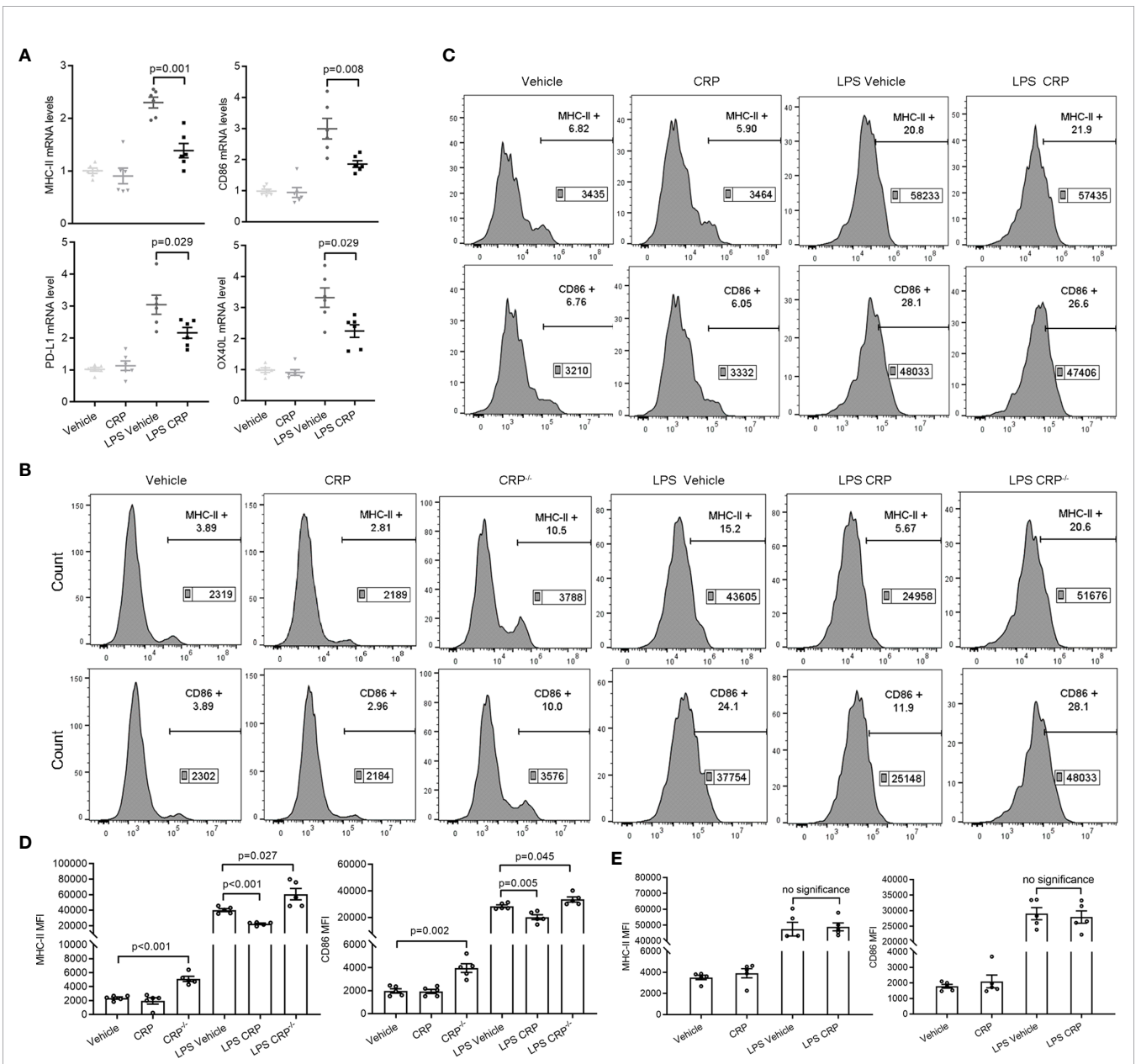


FIGURE 6 | CRP diminishes the antigen presenting ability of moDCs though FcγR2B. **(A)** The mRNA expression levels of MHC-II, CD86, PDL-1, and OX40L were measured in LPS and LPS CRP samples from WT moDCs by qPCR (n = 6). **(B, D)** moDCs were established from WT mice and $CRP^{-/-}$ mice, then incubated with or without CRP and LPS. The expression of MHC-II and CD86 was analyzed by flow cytometry and the MFI was recorded (n = 4). **(C, E)** moDCs were established from $FcγR2B^{-/-}$ mice and cultured with or without CRP and LPS, then the expression of MHC-II and CD86 was analyzed by flow cytometry and the MFI was recorded (n = 4). Data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant. ns, no significance.

from CRP$^{-/-}$ mice had a higher expression of MHC-II and CD86 than from WT mice (**Figures 6B, D**). Next, we used FcγR2B$^{-/-}$ mice to further assess whether FcγR2B mediates the attenuation of antigen presentation ability of moDCs by CRP. The expression of MHC-II and CD86 had no apparent difference in LPS and CRP-treated samples compared to LPS alone (**Figures 6C, E**). Taken together, these results indicated that CRP attenuated the antigen presentation ability of CNS infiltratory moDCs by inhibiting the expression of MHC-II and CD86, and this process was mediated by FcγR2B expressed on moDCs.

NF-κB and ERK Signaling Pathways Involve in Suppressing the Expression of MHC-II and CD86 in moDCs by CRP

Many previous studies have substantiated unequivocally that NF-κB is a crucial regulator of DCs for antigen presentation and therefore is involved in DC maturation (23, 48, 49). ERK is a another signaling molecule that contributes to DCs' survival and maturation through increasing the TNF-α production (50). To delineate the signaling pathways underlying the observed effects of CRP on antigen presentation ability of DCs *via* FcγR2B, moDCs were established from WT mice, CRP$^{-/-}$ mice, and FcγR2B$^{-/-}$ mice, then the expressions of NF-κB (p65) and ERK were evaluated by WB. In WT mice, WB and quantitative analysis showed a significant decrease of NF-κB and phosphorylated ERK in LPS and CRP stimulated samples when compared to LPS stimulation alone, but there was no difference in vehicle and CRP samples (**Figure 7A**). Opposite results were obtained by using moDCs from WT mice and CRP$^{-/-}$ mice; a higher NF-κB and phosphorylated ERK expression in CRP$^{-/-}$ mice than in WT mice (**Figure 7B**) indicated that CRP played an important role in maintaining the moderate activation of antigen presenting cells. In FcγR2B$^{-/-}$ mice, the NF-κB and phosphorylated ERK did not differ significantly between LPS and LPS CRP samples (**Figure 7C**), presumably because the absence of FcγR2B prevented CRP from interfering with DCs' antigen presentation. Overall, our results indicated that NF-κB and ERK signaling were involved in FcγR2B-mediated effects of CRP on antigen presentation and Th17 response.

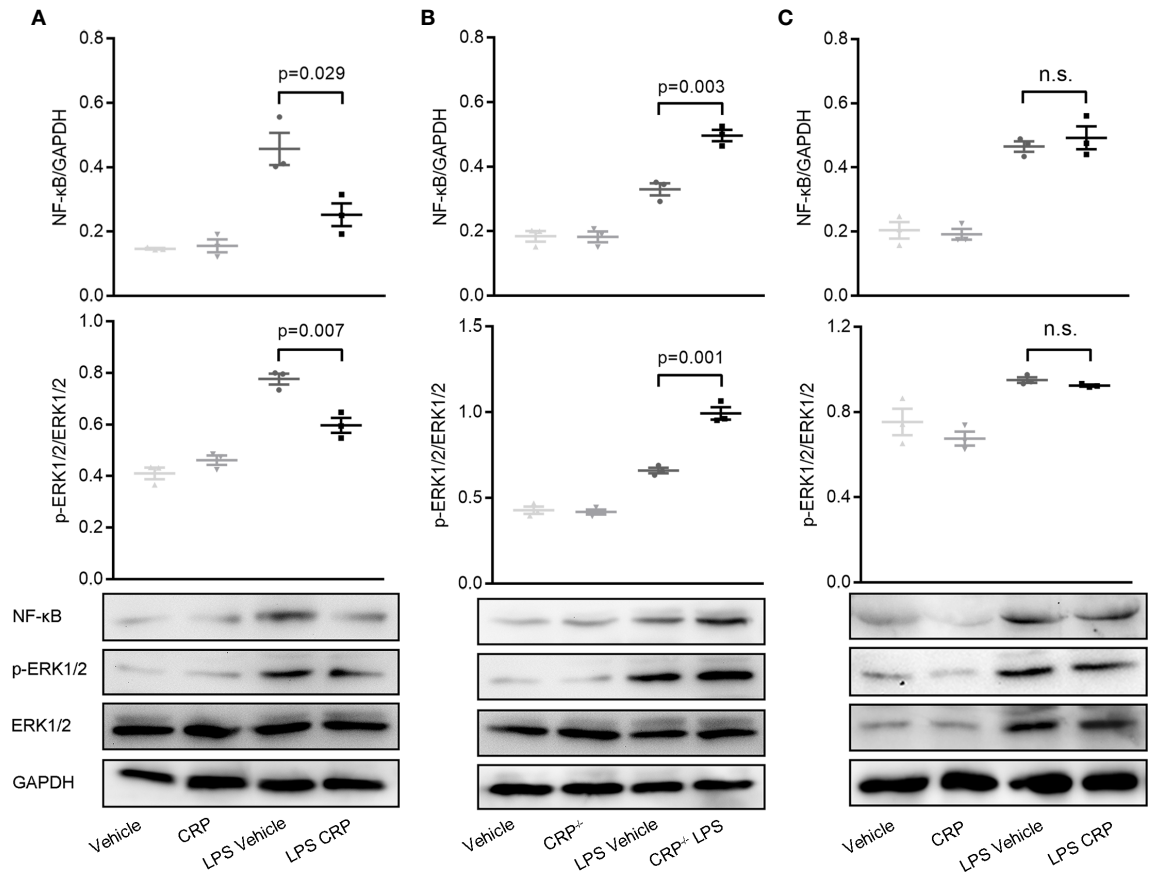


FIGURE 7 | NF-κB and ERK signaling is involved in decreasing the antigen presenting ability of moDCs by CRP.**(A)** Western Blot and quantitative analyses of NF-κB and p-ERK in moDCs from WT mice with or without LPS and CRP (n = 3). **(B)** Western Blot and quantitative analyses of NF-κB and p-ERK in moDCs from WT mice and CRP$^{-/-}$ mice with or without LPS and CRP (n = 3). **(C)** Western Blot and quantitative analyses of NF-κB and p-ERK in moDCs from FcγR2B$^{-/-}$ mice with or without LPS and CRP (n = 3). Data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant.

DISCUSSION

In a previous study, we reported that CRP modulates Th1 and Th2 responses directly by binding to naïve T cells, but there was no effect observed on Th17 response at that time. In this paper, we focused on whether CRP participates in the Th17 response and explored the indirect regulation of CRP on Th17 *via* APCs. Initially, the MOG-induced EAE model was utilized to prove the Th17 response was suppressed *in vivo* with CRP injection, then we used splenocytes and $CD4^+$ T cells to distinguish the difference in Th17 and Th1 response *in vitro*, further speculating that a potential possible of CRP is to act on Th17 response indirectly through APCs. Secondly, we analyzed the immune cell composition in the blood and CNS during the peak of EAE and found that apart from T cells, the moDCs were the main immune cells infiltrating into the CNS, which provides the possibility of CRP to participate in Th17 regulation. Thirdly, FcγR2B had been reported to have a crucial role in EAE regulation of CRP, so $Fc\gamma R2B^{-/-}$ mice were used to verify that CRP participates in Th17 response regulation by FcγR2B *in vivo* and *in vitro*. Finally, the antigen presenting molecules were screened in moDCs from WT mice, which could be influenced by CRP treatment, and NF-κB and ERK signaling was proved to be involved in this process.

Although many previous studies have reported the regulatory role of CRP on normal DCs (27, 42, 51, 52), the studies of CRP on monocyte derived DCs were limited and controversial (24, 28), and their concern with DCs was only because the DCs could express the CRP receptors, but in our study, we focused on DCs because we had solid data to demonstrate that moDCs are the main immune cells infiltrating the CNS to elicit an indirect T cell response in EAE. Moreover, we concentrated on the regulation of CRP on Th17 in EAE; this is very different from previous studies. Our data showed clearly that Th17 and Th1 responses were impaired when the antigen presenting molecule expressions were inhibited by CRP; previous studies did not investigate $CD4^+$ T cell subtypes, but instead only evaluated T cell proliferation. Furthermore, we have more comprehensively explored the mechanism. WT mice, $CRP^{-/-}$ mice, and $Fc\gamma R2B^{-/-}$ mice were used to demonstrate the importance of CRP on the antigen presenting ability of moDCs, and finally NF-κB and ERK signaling and the FcγR2B receptor were confirmed to be involved in this process.

Most important, we verified that moDCs are the main antigen presenting cells in EAE, and we described a precise pathway for inflammatory immune cell infiltration during EAE development, from bone marrow hematopoietic stem cells to blood monocytes to CNS DCs. More concretely, when the mice were immunized with MOG in complete Freund's adjuvant, the blood monocytes were increased sharply and implicated as essential players in defense against microbial pathogens; then they were activated and differentiated into macrophages and DCs, infiltrating the CNS. Meanwhile, in CRP-treated mice, CRP was injected intraperitoneally into mice and absorbed into the blood through the capillaries, where these immune cells could be primed by CRP, thereby influencing monocyte differentiation and maturation. Eventually these immune cells infiltrate the CNS and trigger specific $CD4^+$ T cell responses. Nevertheless, these circulating blood monocytes usually descend from self-renewing hematopoietic stem cells that initiate myeloid differentiation (53, 54) (**Figure 8**).

During all the data collection, Th17 and Th1 relevant data were always harvested together, because both Th1 and Th17 subsets are the main mediators of EAE (55), and we wanted to know all the contributions of CRP on EAE remission. Moreover, we proposed that the regulation of CRP on Th17 requires the aid of APCs, while for Th1 modulation, direct and indirect regulations work together, so there is a difference of CRP on Th17 and Th1 responses. Furthermore, we should note that the indirect regulation is not specific to Th17 response, but also affects Th1 and Th2 responses, depending on the disease state and the immune microenvironment at that time. In this regard, all T cell mediated autoimmune diseases like SLE and RA could theoretically be improved by CRP with the help of APCs. Overall,

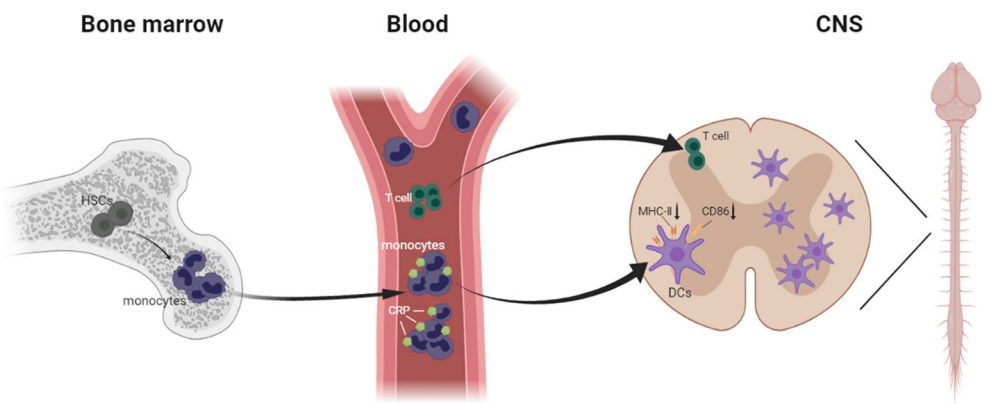


FIGURE 8 | Schematic model demonstrating immune cells infiltrating from the bone marrow to the CNS in EAE.

the indirect APC-mediated pathway presented in this paper enriches our understanding of how CRP regulates T cells and leads to EAE remission.

In conclusion, although CRP is an innate molecule, more and more studies focus on its acquired immune function in recent years. Our present work expands on the existing research, and we put forward a new way for CRP participating in the regulation of Th17 response in EAE, an effect which depends on the APCs and is mediated through FcγR2B, as well as NF-κB and ERK signaling pathways. Our study adds a new dimension to understand the multi-faceted effects of CRP in EAE remission, which suggests that CRP may be a novel drug target for the fundamental prevention and treatment of MS and other T-cell mediated autoimmune diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Center of Xi'an Jiaotong University.

AUTHOR CONTRIBUTIONS

LZ supervised the project. LZ and YZ designed the research. Z-YS and YZ performed most of the experiments. KW, WL, M-JG, FW, and YZ carried out part of the experiments. LZ, Z-YS, and MP analyzed the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the following grants: National Natural Science Foundation of China (Nos. 82070876, 31700685, 31800852), Scientific Research Fund of Xi'an Jiaotong University (No. XJJ2018126), China Postdoctoral Science Foundation (No. 2020M673421), and Project of Shaanxi Key Research and Development Plan (No. 2020SF-082).

ACKNOWLEDGMENTS

We are thankful for the financial support from Prof. Shang-Rong Ji and Yi Wu.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.589200/full#supplementary-material

Supplementary Figure 1 | CRP alleviates MOG-immunized EAE severity both in WT mice and FcγR2B-/- mice. **(A)** The clinical sores of EAE were recorded daily in WT mice and FcγR2B-/- mice respectively. **(B)** The body weight was recorded daily in WT mice and FcγR2B-/- mice, which is consistent with EAE severity. **(C)** The degree of demyelination of each group was shown in LFB staining. **(D)** HE staining displayed the infiltration of immune cells for each groups. Data are presented as mean ± SEM, $p < 0.05$ was considered statistically significant.

Supplementary Figure 2 | CRP effects the antigen presenting ability of moDCs from WT mice. **(A)** Antigen presenting molecular MHC-II, CD86, CD80, CD70, COSL-1, PD-L1, PD-L2, OX40L, BTLA, HEVM, SLAM and 4-1BBL were screened by qPCR (n = 6). **(B)** Flow cytometry of PD-L1 was no apparent difference between LPS and LPS CRP treated samples (n = 4). **(C)** Flow cytometry of OX40l was unchanged between LPS and LPS CRP treated samples (n = 4). Data are presented as mean ± SEM, $p < 0.05$ was considered statistically significant.

REFERENCES

1. Frohman EM, Racke MK, Raine CS. Multiple sclerosis–the plaque and its pathogenesis. *New Engl J Med* (2006) 354(9):942–55. doi: 10.1056/NEJMra052130
2. Sospedra M, Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* (2005) 23:683–747. doi: 10.1146/annurev.immunol.23.021704.115707
3. Nylander A, Hafler DA. Multiple sclerosis. *J Clin Invest* (2012) 122(4):1180–8. doi: 10.1172/JCI58649
4. Booss J, Esiri MM, Tourtellotte WW, Mason DY. Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. *J Neurological Sci* (1983) 62(1-3):219–32. doi: 10.1016/0022-510X(83)90201-0
5. Axisa PP, Hafler DA. Multiple sclerosis: genetics, biomarkers, treatments. *Curr Opin Neurology* (2016) 29(3):345–53. doi: 10.1097/WCO.0000000000000319
6. Marson A, Housley WJ, Hafler DA. Genetic basis of autoimmunity. *J Clin Invest* (2015) 125(6):2234–41. doi: 10.1172/JCI78086
7. Rostami A, Ciric B. Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. *J Neurological Sci* (2013) 333(1-2):76–87. doi: 10.1016/j.jns.2013.03.002
8. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol* (2009) 183(11):7169–77. doi: 10.4049/jimmunol.0901906
9. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest* (2003) 111(12):1805–12. doi: 10.1172/JCI18921
10. Verma S, Szmitko PE, Yeh ET. C-reactive protein: structure affects function. *Circulation* (2004) 109(16):1914–7. doi: 10.1161/01.CIR.0000127085.32999.64
11. Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: pentraxins as a paradigm. *Annu Rev Immunol* (2010) 28:157–83. doi: 10.1146/annurev-immunol-030409-101305
12. Du Clos TW. Pentraxins: structure, function, and role in inflammation. *ISRN Inflammation* (2013) 2013:379040. doi: 10.1155/2013/379040
13. Szalai AJ, Nataf S, Hu XZ, Barnum SR. Experimental allergic encephalomyelitis is inhibited in transgenic mice expressing human C-reactive protein. *J Immunol* (2002) 168(11):5792–7. doi: 10.4049/jimmunol.168.11.5792
14. Gaitonde S, Samols D, Kushner I. C-reactive protein and systemic lupus erythematosus. *Arthritis Rheumatism* (2008) 59(12):1814–20. doi: 10.1002/art.24316
15. Veldhuijzen van Zanten JJ, Ring C, Carroll D, Kitas GD. Increased C reactive protein in response to acute stress in patients with rheumatoid arthritis. *Ann Rheumatic Diseases* (2005) 64(9):1299–304. doi: 10.1136/ard.2004.032151
16. Zhang L, Liu SH, Wright TT, Shen ZY, Li HY, Zhu W, et al. C-reactive protein directly suppresses Th1 cell differentiation and alleviates experimental autoimmune encephalomyelitis. *J Immunol* (2015) 194(11):5243–52. doi: 10.4049/jimmunol.1402909
17. Damsker JM, Hansen AM, Caspi RR. Th1 and Th17 cells: adversaries and collaborators. *Ann New York Acad Sci* (2010) 1183:211–21. doi: 10.1111/j.1749-6632.2009.05133.x

18. Yang YH, Weiner J, Liu Y, Smith AJ, Huss DJ, Winger R, et al. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J Exp Med* (2009) 206(7):1549–64. doi: 10.1084/jem.20082584
19. Croft SM, Mortensen RF, Gewurz H. Binding of C-reactive protein to antigen-induced but not mitogen-induced T lymphoblasts. *Science.* (1976) 193 (4254):685–7. doi: 10.1126/science.1085034
20. Mortensen RF, Osmand AP, Gewurz H. Effects on C-reactive protein on the lymphoid system. I. Binding to thymus-dependent lymphocytes and alteration of their functions. *J Exp Med* (1975) 141(4):821–39. doi: 10.1084/jem.141.4.821
21. Amigorena S, Bonnerot C, Choquet D, Fridman WH, Teillaud JL. Fc gamma RII expression in resting and activated B lymphocytes. *Eur J Immunol* (1989) 19(8):1379–85. doi: 10.1002/eji.1830190805
22. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* (2008) 8(1):34–47. doi: 10.1038/nri2206
23. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell.* (2001) 106(3):255–8. doi: 10.1016/s0092-8674(01)00449-4
24. Van Vre EA, Bult H, Hoymans VY, Van Tendeloo VF, Vrints CJ, Bosmans JM. Human C-reactive protein activates monocyte-derived dendritic cells and induces dendritic cell-mediated T-cell activation. *Arteriosclerosis Thrombosis Vasc Biol* (2008) 28(3):511–8. doi: 10.1161/ATVBAHA.107.157016
25. Hilhorst M, Shirai T, Berry G, Goronzy JJ, Weyand CM. T cell-macrophage interactions and granuloma formation in vasculitis. *Front Immunol* (2014) 5:432. doi: 10.3389/fimmu.2014.00432
26. Richter A, Nebe T, Kattermann R, Trede M. [Immune paralysis in acute pancreatitis–HLA-DR antigen expression on CD14+DR+ monocytes]. *Langenbecks Archiv fur Chirurgie* (1996) 381(1):38–41. doi: 10.1007/bf00184253
27. Jimenez RV, Wright TT, Jones NR, Wu J, Gibson AW, Szalai AJ. C-Reactive Protein Impairs Dendritic Cell Development, Maturation, and Function: Implications for Peripheral Tolerance. *Front Immunol* (2018) 9:372. doi: 10.3389/fimmu.2018.00372
28. Zhang R, Becnel L, Li M, Chen C, Yao Q. C-reactive protein impairs human CD14+ monocyte-derived dendritic cell differentiation, maturation and function. *Eur J Immunol* (2006) 36(11):2993–3006. doi: 10.1002/eji.200635207
29. Zhang L, Shen ZY, Wang K, Li W, Shi JM, Osoro EK, et al. C-reactive protein exacerbates epithelial-mesenchymal transition through Wnt/beta-catenin and ERK signaling in streptozocin-induced diabetic nephropathy. *FASEB J Off Publ Fed Am Societies Exp Biol* (2019) 33(5):6551–63. doi: 10.1096/fj.201801865RR
30. Zhang L, Li HY, Li W, Shen ZY, Wang YD, Ji SR, et al. An ELISA Assay for Quantifying Monomeric C-Reactive Protein in Plasma. *Front Immunol* (2018) 9:511. doi: 10.3389/Fimmu.2018.00511
31. Liu B, Gu Y, Pei S, Peng Y, Chen J, Pham LV, et al. Interleukin-1 receptor associated kinase (IRAK)-M -mediated type 2 microglia polarization ameliorates the severity of experimental autoimmune encephalomyelitis (EAE). *J Autoimmunity* (2019) 102:77–88. doi: 10.1016/j.jaut.2019.04.020
32. McQualter JL, Darwiche R, Ewing C, Onuki M, Kay TW, Hamilton JA, et al. Granulocyte macrophage colony-stimulating factor: a new putative therapeutic target in multiple sclerosis. *J Exp Med* (2001) 194(7):873–82. doi: 10.1084/jem.194.7.873
33. Chong WP, Horai R, Mattapallil MJ, Silver PB, Chen J, Zhou R, et al. IL-27p28 inhibits central nervous system autoimmunity by concurrently antagonizing Th1 and Th17 responses. *J Autoimmunity* (2014) 50:12–22. doi: 10.1016/j.jaut.2013.08.003
34. Dardalhon V, Korn T, Kuchroo VK, Anderson AC. Role of Th1 and Th17 cells in organ-specific autoimmunity. *J Autoimmunity* (2008) 31(3):252–6. doi: 10.1016/j.jaut.2008.04.017
35. Codarri L, Gyulveszi G, Tosevski V, Hesske L, Fontana A, Magnenat L, et al. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* (2011) 12(6):560–7. doi: 10.1038/ni.2027
36. Yamasaki R, Lu H, Butovsky O, Ohno N, Rietsch AM, Cialic R, et al. Differential roles of microglia and monocytes in the inflamed central nervous system. *J Exp Med* (2014) 211(8):1533–49. doi: 10.1084/jem.20132477
37. Heuertz RM, Tricomi SM, Ezekiel UR, Webster RO. C-reactive protein inhibits chemotactic peptide-induced p38 mitogen-activated protein kinase activity and human neutrophil movement. *J Biol Chem* (1999) 274(25):17968–74. doi: 10.1074/jbc.274.25.17968
38. Meng S, Zhang L, Zhao L, Fang YR, Fujimoto T, Hirano S, et al. Effects of C-reactive protein on CC chemokine receptor 2-mediated chemotaxis of monocytes. *DNA Cell Biol* (2012) 31(1):30–5. doi: 10.1089/dna.2010.1178
39. Tacken PJ, de Vries IJ, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. *Nat Rev Immunol* (2007) 7(10):790–802. doi: 10.1038/nri2173
40. Amigorena S. Fc gamma receptors and cross-presentation in dendritic cells. *J Exp Med* (2002) 195(1):F1–3. doi: 10.1084/jem.20011925
41. Regnault A, Lankar D, Lacabanne V, Rodriguez A, Thery C, Rescigno M, et al. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* (1999) 189(2):371–80. doi: 10.1084/jem.189.2.371
42. Hu XZ, Wright TT, Jones NR, Ramos TN, Skibinski GA, McCrory MA, et al. Inhibition of Experimental Autoimmune Encephalomyelitis in Human C-Reactive Protein Transgenic Mice Is FcgammaRIIB Dependent. *Autoimmune Diseases* (2010) 2011:484936. doi: 10.4061/2011/484936
43. Nowatzky J, Manches O, Khan SA, Godefroy E, Bhardwaj N. Modulation of human Th17 cell responses through complement receptor 3 (CD11b/CD18) ligation on monocyte-derived dendritic cells. *J Autoimmun* (2018) 92:57–66. doi: 10.1016/j.jaut.2018.05.005
44. Baleeiro RB, Walden P. Immature human DCs efficiently translocate endocytosed antigens into the cytosol for proteasomal processing. *Mol Immunol* (2017) 88:148–54. doi: 10.1016/j.molimm.2017.06.028
45. Wolk K, Docke WD, von Baehr V, Volk HD, Sabat R. Impaired antigen presentation by human monocytes during endotoxin tolerance. *Blood.* (2000) 96(1):218–23. doi: 10.1182/blood.V96.1.218
46. Creusot RJ, Postigo-Fernandez J, Teteloshvili N. Altered Function of Antigen-Presenting Cells in Type 1 Diabetes: A Challenge for Antigen-Specific Immunotherapy? *Diabetes.* (2018) 67(8):1481–94. doi: 10.2337/db17-1564
47. Ferragine CE, Walls CD, Davies SJ. Modulation of innate antigen-presenting cell function by pre-patent schistosome infection. *PloS Neglected Trop Diseases* (2013) 7(3):e2136. doi: 10.1371/journal.pntd.0002136
48. Yoshimura S, Bondeson J, Foxwell BM, Brennan FM, Feldmann M. Effective antigen presentation by dendritic cells is NF-kappaB dependent: coordinate regulation of MHC, co-stimulatory molecules and cytokines. *Int Immunol* (2001) 13(5):675–83. doi: 10.1093/intimm/13.5.675
49. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* (2000) 18:767–811. doi: 10.1146/annurev.immunol.18.1.767
50. Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med* (1998) 188(11):2175–80. doi: 10.1084/jem.188.11.2175
51. Mold C, Clos TW. C-reactive protein inhibits plasmacytoid dendritic cell interferon responses to autoantibody immune complexes. *Arthritis Rheumatism* (2013) 65(7):1891–901. doi: 10.1002/art.37968
52. Thomas-Rudolph D, Du Clos TW, Snapper CM, Mold C. C-reactive protein enhances immunity to Streptococcus pneumoniae by targeting uptake to Fc gamma R on dendritic cells. *J Immunol* (2007) 178(11):7283–91. doi: 10.4049/jimmunol.178.11.7283
53. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol* (2008) 26:421–52. doi: 10.1146/annurev.immunol.26.021607.090326
54. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science* (2010) 327 (5966):656–61. doi: 10.1126/science.1178331
55. Dhaeze T, Tremblay L, Lachance C, Peelen E, Zandee S, Grasmuck C, et al. CD70 defines a subset of proinflammatory and CNS-pathogenic TH1/TH17 lymphocytes and is overexpressed in multiple sclerosis. *Cell Mol Immunol* (2019) 16(7):652–65. doi: 10.1038/s41423-018-0198-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 June 2021
doi: 10.3389/fimmu.2021.699128

Regulatory Dendritic Cells Induced by Bendamustine Are Associated With Enhanced Flt3 Expression and Alloreactive T-Cell Death

*Megan S. Molina[1,2], Emely A. Hoffman[1], Jessica Stokes[1], Nicole Kummet[1,3], Kyle A. Smith[4,5], Forrest Baker[1,5], Tiffany M. Zúñiga[5], Richard J. Simpson[1,2,5,6] and Emmanuel Katsanis[1,2,6,7,8]**

[1] Department of Pediatrics, University of Arizona, Tucson, AZ, United States, [2] Department of Immunobiology, University of Arizona, Tucson, AZ, United States, [3] Department of Molecular & Cellular Biology, University of Arizona, Tucson, AZ, United States, [4] Department of Physiology, University of Arizona, Tucson, AZ, United States, [5] Department of Nutritional Sciences, University of Arizona, Tucson, AZ, United States, [6] The University of Arizona Cancer Center, Tucson, AZ, United States, [7] Department of Medicine, University of Arizona, Tucson, AZ, United States, [8] Department of Pathology, University of Arizona, Tucson, AZ, United States

The growth factor Flt3 ligand (Flt3L) is central to dendritic cell (DC) homeostasis and development, controlling survival and expansion by binding to Flt3 receptor tyrosine kinase on the surface of DCs. In the context of hematopoietic cell transplantation, Flt3L has been found to suppress graft-versus-host disease (GvHD), specifically *via* host DCs. We previously reported that the pre-transplant conditioning regimen consisting of bendamustine (BEN) and total body irradiation (TBI) results in significantly reduced GvHD compared to cyclophosphamide (CY)+TBI. Pre-transplant BEN+TBI conditioning was also associated with greater Flt3 expression among host DCs and an accumulation of pre-cDC1s. Here, we demonstrate that exposure to BEN increases Flt3 expression on both murine bone marrow-derived DCs (BMDCs) and human monocyte-derived DCs (moDCs). BEN favors development of murine plasmacytoid DCs, pre-cDC1s, and cDC2s. While humans do not have an identifiable equivalent to murine pre-cDC1s, exposure to BEN resulted in decreased plasmacytoid DCs and increased cDC2s. BEN exposure and heightened Flt3 signaling are associated with a distinct regulatory phenotype, with increased PD-L1 expression and decreased ICOS-L expression. BMDCs exposed to BEN exhibit diminished pro-inflammatory cytokine response to LPS and induce robust proliferation of alloreactive T-cells. These proliferative alloreactive T-cells expressed greater levels of PD-1 and underwent increased programmed cell death as the concentration of BEN exposure increased. Alloreactive $CD4^+$ T-cell death may be attributable to pre-cDC1s and provides a potential mechanism by which BEN+TBI conditioning limits GvHD and yields T-cells tolerant to host antigen.

Keywords: dendritic cell, Flt3, bendamustine, Alloreactivity, Regulatory DC

OPEN ACCESS

Edited by:
Fang Zhou,
CAS Lamvac Biotech Co., Ltd., China

Reviewed by:
Yaping Sun,
University of Michigan, United States
Yuanyuan Tian,
Children's Hospital of Soochow University, China
Cynthia Giver,
Emory University, United States

***Correspondence:**
Emmanuel Katsanis
ekatsani@arizona.edu
orcid.org/0000-0003-1466-6965

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *22 April 2021*
Accepted: *14 June 2021*
Published: *24 June 2021*

Citation:
Molina MS, Hoffman EA, Stokes J, Kummet N, Smith KA, Baker F, Zúñiga TM, Simpson RJ and Katsanis E (2021) Regulatory Dendritic Cells Induced by Bendamustine Are Associated With Enhanced Flt3 Expression and Alloreactive T-Cell Death. Front. Immunol. 12:699128. doi: 10.3389/fimmu.2021.699128

INTRODUCTION

Fms-like tyrosine kinase 3 (Flt3) (aka CD135, Flk2, STK1) is a receptor tyrosine kinase that binds the growth factor Flt3 Ligand (Flt3L) (1–5). Flt3 is expressed by early hematopoietic cells and controls their survival and expansion (3, 4, 6, 7). Flt3 expression is lost as hematopoietic precursors differentiate, but expression is maintained on dendritic cells (DCs) through their terminal differentiation (8, 9). Flt3 signaling is crucial to the homeostasis and development of steady state DCs (3, 10–17). Given the critical role of DCs in graft-versus-host disease (GvHD) (18, 19) and graft-versus-leukemia (GvL) (20), Flt3L has been investigated by numerous groups in the context of hematopoietic stem cell transplantation (HSCT). Administration of Flt3L to recipients *prior* to transplant significantly reduces GvHD, an effect largely attributed to increased numbers of host CD8α$^+$ type 1 conventional DCs (cDC1s) capable of inducing clonal deletion of alloantigen-specific T-cells (21–23).

Previous work from our laboratory using murine bone marrow transplantation (BMT) models found that bendamustine (BEN) supplemented with total body irradiation (TBI) conditioning results in significantly reduced GvHD and improved survival compared to cyclophosphamide (CY)+TBI, the standard regimen used in cases of acute lymphoblastic leukemia (ALL) (24–27). BEN is a cytotoxic alkylating agent with diverse immunomodulatory properties (24–26, 28–33). Importantly, BEN+TBI conditioning yields donor T-cells that are tolerant to host, while preserving T-cell-dependent GvL (26). BEN+TBI also results in a more favorable host DC composition at the time of transplant, with increased frequencies of cDC1s, most substantially pre-cDC1s (27). Host DCs from BEN-treated mice also display greater Flt3 expression compared to CY-treated DCs (27). It remains unclear if increased Flt3 expression is a direct effect of BEN. Given the clear advantage of enhanced Flt3 signaling in host DCs in the context of transplantation, this warranted further investigation. Moreover, it is not understood whether enhanced Flt3 expression alters DC development or function in the same manner as administration of exogenous Flt3L.

Here we investigate the ability of BEN to directly induce increased Flt3 expression in murine bone marrow (BM) progenitors and DCs, and examine the effect of BEN exposure on dendropoiesis in murine and human DCs *in vitro*. We further investigate how murine DCs exposed to BEN mature in response to TLR activation and stimulate alloreactive T-cell responses. Overall, our results demonstrate that BEN elicits a regulatory program in DCs, associated with increased Flt3 signaling. This "regulatory" program is exemplified by increased expression of inhibitory co-stimulatory molecules (PD-L1), a minimal pro-inflammatory response to lipopolysaccharide (LPS) stimulation, and robust activation-induced death of alloreactive CD4$^+$ T-cells. This work highlights the capacity of Flt3L-driven DCs to regulate alloreactive CD4$^+$ T-cell responses in a way that is highly advantageous for GvHD and may preserve GvL by sparing alloreactive CD8$^+$ T-cells.

METHODS

Mice

All strains of mice used (BALB/c and C57BL/6) were age-matched 6-10-week-old females purchased from The Jackson Laboratory. Mice were housed in specific pathogen-free conditions and cared for according to the guidelines of the University of Arizona's Institutional Animal Care and Use Committee.

Drug Preparation and Administration

BEN (SelleckChem) was reconstituted and diluted for *in vivo* administration as previously described (24–27). AC220 (SelleckChem) and JSI-124 (Santa Cruz Biotechnology) were reconstituted in DMSO (Sigma-Aldrich). For *in vitro* studies, stock solutions of drugs were diluted in complete media (CM) consisting of RPMI-1640 with 10% FBS, 1% Sodium Pyruvate, 1% MEM NEAA, and 100 U/mL penicillin-streptomycin to their final concentrations.

Murine Bone Marrow-Derived DCs (BMDCs)

Murine bone marrow (**BM**) cells were collected, red blood cells were lysed with Pharm Lyse (BD Biosciences), and 3x10^6 BM cells were plated per well in 6-well plates at a concentration of 10^6/mL. BM was cultured at 37°C and 5% CO_2 in CM containing 200 ng/mL of rhFlt3L (Miltenyi Biotec) with or without drugs (bendamustine, AC220, or JSI-124). After 4 hours of culture, all media were washed out, BM cells were washed with PBS and again cultured in CM containing 200 ng/mL of rhFlt3L. Culture media was replenished on day 3 and 5. LPS (Sigma-Aldrich) was added on day 5 of culture for 18 hours at a final concentration of 1 μg/mL. Individual wells of BMDCs were collected on day 6.

Absolute Counts and Viability

BMDCs were resuspended in PBS and analyzed by MACSQuant Analyzer 10 (Miltenyi Biotec) to determine absolute counts and viability by Propidium Iodide staining.

Flow Cytometry

Cells were washed in flow buffer (PBS with 0.5% FBS), incubated with anti-mouse or anti-human Fc Block (Thermo Fisher Scientific), and flow cytometry was performed as previously reported (24–27, 34). Intracellular staining of human moDCs was performed using TruePhos Perm Buffer (Biolegend). All antibodies used for flow cytometry are listed in **Table 1**. Fluorescence data were collected using an LSRFortessa cell analyzer (BD Biosciences) and analyzed using FlowJo 2 (Tree Star). Total DCs were defined as CD11c$^+$. Plasmacytoid DCs (pDCs) were defined as CD11c$^+$B220$^+$. Conventional DCs (cDCs) were defined as CD11c$^+$ B220$^-$. Type 1 conventional DCs (cDC1s) were defined as CD11c$^+$B220$^-$CD8α$^+$ and CD11c$^+$B220$^-$CD103$^+$. Type 2 conventional DCs (cDC2s) were defined as CD11c$^+$B220$^-$SIRPα$^+$. Pre-cDC1s were defined as CD11c$^+$B220$^-$CD24^{high}CD8α$^-$. Pre-cDC2s were defined as CD11c$^+$B220$^-$SIRPα$^+$CD24^{mid}.

TABLE 1 | Antibodies used for flow cytometry.

Antibody	Clone(s)	Vendor
Anti-mouse B220 Brilliant Violet 510	RA3-6B2	Biolegend
Anti-mouse CCL2 PE	2H5	Biolegend
Anti-mouse CCL5 PE-Cyanine7	2E9	Biolegend
Anti-mouse CD4 APC/Cy7	GK1.5	Biolegend
Anti-mouse CD8α PE-Cyanine7	53-6.7	Thermo Fisher
Anti-mouse CD11c FITC	N418	Miltenyi Biotec
Anti-mouse CD11c VioBlue	REA754	Miltenyi Biotec
Anti-mouse CD24 Pacific Blue	M1/69	Biolegend
Anti-mouse CD24 PE-Dazzle 594	M1/69	Biolegend
Anti-mouse CD69 PE/Cyanine5	H1.2F3	Thermo Fisher
Anti-mouse CD70 PerCP-eFluor710	FR70	Thermo Fisher
Anti-mouse CD80 APC	16-10A1	Biolegend
Anti-mouse CD86 AlexaFluor700	GL-1	Biolegend
Anti-mouse CD103 PE	2E7	Thermo Fisher
Anti-mouse CD135 PE-CF594	A2F10.1	BD Biosciences
Anti-mouse H2Kb PerCP-eFluor710	AF6-88.5.5.3	Thermo Fisher
Anti-mouse ICOS VioGreen	REA192	Miltenyi Biotec
Anti-mouse ICOSL PE	HK5.3	Biolegend
Anti-mouse IL-6 APC	MP5-20F3	Biolegend
Anti-mouse IL-10 APC-Cyanine7	JES5-16E3)	Biolegend
Anti-mouse IDO-1 AlexaFluor 647	2E2/IDO1	Biolegend
Anti-mouse PD-1 APC	29F.1A12	Biolegend
Anti-mouse PD-L1 PE/Dazzle594	10F.9G2	Biolegend
Anti-mouse PIR-B APC	10-1-PIR	Thermo Fisher
Anti-mouse SIRPα APC-Cyanine7	P84	Biolegend
Anti-mouse TIM-3 PE	REA602	Miltenyi Biotec
Anti-mouse TNFα Brilliant Violet 510	MP6-XT22	Biolegend
Anti-human AXL PE-Cyanine7	DS7HAXL	Thermo Fisher
Anti-human BDCA1 PE-Vio615	REA694	Miltenyi Biotec
Anti-human BDCA3 APC-Vio770	REA774	Miltenyi Biotec
Anti-human BDCA3 Brilliant Violet 421	M80	Biolegend
Anti-human CD11c AlexaFluor 488	3.9	Biolegend
Anti-human CD14 Brilliant Violet 421	MPHIP9	BD Biosciences
Anti-human Clec9a PE	8F9	Biolegend
Anti-human Lineage (CD3/14/19/20/56) Cocktail APC	UCHT1; HCD14; HIB19; 2H7; HCD56	Biolegend
Anti-human STAT3 Phospho(Tyr705) PerCP/Cyanine5.5	13A3-1	Biolegend
Isotype BV510 Rat IgG1,k	RTK2071	Biolegend
Isotype APC Rat IgG1	RTK2071	Biolegend
Isotype PE Armenian Hamster IgG	HTK888	Biolegend
Isotype PE-Cyanine7 Mouse IgG2b,k	MPC-11	Biolegend
Isotype APC-Cyanine7 Rat IgG2b	RTK4530	Biolegend

ELISAs

Cytokines in culture supernatants were measured with ELISA kits (R&D Systems).

Intracellular Cytokine Staining

FL-BMDCs were LPS-activated on day 6 for 3-4 hours. Protein transport inhibitors GolgiStop (Thermo Fisher) and GolgiPlug (Thermo Fisher) were incubated with FL-BMDCs for 4-6 hours. After Fc block, FL-BMDCs were fixed and stained using Fixation Buffer (Biolegend) and Intracellular Staining Perm Wash Buffer (Biolegend). Antibodies used for flow cytometry are listed in **Table 1**.

Mixed Leukocyte Reaction (MLR)

Unstimulated FL-BMDCs were counted and enriched for live cells using EasySep Dead Cell Removal (Annexin V) kit (STEMCELL Technologies). Allogeneic T-cells were isolated from the spleens of naïve C57BL/6 mice using the Pan T-cell isolation kit II (Miltenyi Biotec). Purified T-cells were stained with CellTrace Violet (Invitrogen). Live FL-BMDCs were co-cultured with allogeneic T-cells at a ratio of 1:10 and incubated at 37°C with 7.5% CO_2. T-cells were stimulated with CD3/CD28 DynaBeads (Thermo Fisher Scientific) as a positive control. After 16-24 hours, rIL-2 (PeproTech) was added to each well at a final concentration of 50 IU/mL. After 3-4 days of co-incubation flow cytometry was performed, and data were analyzed using Modfit software (Verity Software House) to determine the proliferation index (PI) of $H2K^{b+}$ T-cells. T-cell death was determined using Propidium Iodide Ready Flow Reagent (Invitrogen).

Human Monocytic-DCs

Healthy human volunteers were recruited as part of an institutional review board (IRB)-approved research protocol. Our protocol for generating human monocytic-DCs (moDC) was adapted from previously reported protocols (35–37). Peripheral blood was collected and whole blood was diluted 1:1

with PBS, layered on top of Ficoll (GE Healthcare Life Sciences), and then centrifuged per the manufacturer's recommendation. $CD14^+$ monocytes were isolated using $CD14^+$ MicroBeads (Miltenyi Biotec) with >97% purity (data not shown), counted, and then re-suspended in RPMI-14 containing 10% FBS, 10% antibiotic-antimycotic solution (ThermoFisher), 500 U/mL rhIL-4 (PeproTech), 800 U/mL rhGM-CSF (PeproTech), and 100 ng/mL rhFlt3L (Miltenyi Biotec). Monocytes were plated into a 6-well plate with 1-1.5x10^6 monocytes per well, the indicated concentration of BEN, and then cultured at 37°C and 5% CO_2. After 4 hours, all BEN-containing media was washed out, cells were washed with PBS and cultured again in the same media at 37°C and 5% CO_2. Media was replenished on day 3 of culture, and moDCs were collected on day 5 of culture for flow cytometry.

qRT-PCR

Samples were saved in PBS and RNAlater (Invitrogen), mRNA was isolated using RNeasy Kit (Qiagen) and then reverse transcribed into cDNA using iScripts reverse transcription supermix kit (Bio-Rad). Quantitative rtPCR was performed using Sso Advanced universal probes supermix (Bio-Rad) on a LightCycler 96 thermocycler (Roche) named Laurel. The appropriate concentration of cDNA was titrated for each TaqMan probe (Applied Biosystems), listed in **Table 2**. The $2^{-\Delta\Delta CT}$ method was used to analyze gene expression levels, normalized for GAPDH expression, as previously described (38, 39).

Statistical Analysis

One-way ANOVA tests and Dunnett's multiple comparisons tests were used to determine significance among absolute counts, percent, and MFI expression. Two-way ANOVA tests and Šidák's multiple comparisons tests were used to determine significance in unstimulated versus LPS-stimulated conditions. P values <0.05 were considered statistically significant.

RESULTS

Dose-Dependent Increase in Flt3 Expression on Murine Bone Marrow Cells *In Vivo* Following BEN Administration

We first sought to determine whether our previous report of increased Flt3 expression *in vivo* was a direct effect of BEN. Mice were given various doses of BEN or vehicle and bone marrow (BM) was collected 48 hours later, reproducing the timing used in our previously published dosing regimens (25–27). There was an anticipated decrease in absolute counts as the dose of BEN increased, but we found no loss of viability of BM cells (data not shown). We observed that the percent expression of Flt3 increased in a dose-dependent manner on total BM cells (**Figure 1A**). We also found a dose-dependent increase in the percent of $CD11c^+$ DCs within the BM compartment (**Figure 1B**) and Flt3 expression on $CD11c^+$ BM cells (**Figure 1C**).

TABLE 2 | Primers used for qRT-PCR.

Target Gene	Taqman Assay ID	Concentration of cDNA used
Mouse Akt1	Mm01331626_m1	5 ng
Mouse Csf2ra	Mm00438331_g1	5 ng
Mouse Csf2rb	Mm00655745_m1	5 ng
Mouse Csf3r	Mm00438334_m1	10 ng
Mouse Flt3	Mm00439016_m1	20 ng
Mouse GAPDH	Mm99999915_g1	2 ng
Mouse Spi1 (PU.1)	Mm00488140_m1	5 ng
Mouse STAT3	Mm01219775_m1	10 ng
Human Akt1	Hs00178289_m1	2 ng
Human GAPDH	Hs02786624_g1	2 ng

BEN Exposure Increases Flt3 Expression on Murine DCs *In Vitro*

We next sought to eliminate physiological variables by utilizing *in vitro* bone marrow-derived DC (BMDC) systems (40–45). Murine BM cells were cultured with Flt3L (FL-BMDCs) in the presence of BEN for just 4 hours to more closely mimic clinical exposure to BEN which has a short half-life of ~40 minutes (46). After exposure to various concentrations of BEN (0 μM, 3 μM, 10 μM, 30 μM, or 100 μM) in culture for 4 hours, BM cells were washed in PBS, then cultured again for the remaining 6 days with Flt3L. As expected, we saw a modest decrease in absolute number (**Figure 1D**) and percent viable FL-BMDCs (**Figure 1E**) as the concentration of BEN increased. We also observed a concentration-dependent increase in percent Flt3 expression among total live $CD11c^+$ FL-BMDCs (**Figure 1F**). The absolute number of $Flt3^+$ $CD11c^+$ BMDCs increases as the concentration of BEN increases (**Figure 1G**) suggesting that BEN is not selectively killing Flt3-negative cells.

BEN Exposure Favors Plasmacytoid, Pre-cDC1, and cDC2 Development

FL-BMDCs generated following 4-hour exposure to BEN were characterized to determine DC composition. As the concentration of BEN increased, the percentage of $CD11c^+$ FL-BMDCs trended upward (**Figure 1H**) while the percentage of pDCs significantly increased (**Figure 1I**) and the percentage of cDCs slightly decreased (**Figure 1J**). We observed a concentration-dependent increase in pre-cDC1s (**Figure 1K**), however we do not observe an increase in $CD8\alpha^+$ cDC1s (**Figure 1L**) and only a slight trend toward increased $CD103^+$ cDC1s (**Figure 1M**). We see a trend toward decreased pre-cDC2s (**Figure 1N**) and an increase in $SIRP\alpha^+$ cDC2s (**Figure 1O**). These results largely match our report on BEN's effect on DC composition *in vivo* (27), indicating that BEN promotes DC development in favor of pDCs, pre-cDC1s, and cDC2s.

BEN Exposure Alters Co-Stimulatory and Co-Inhibitory Molecule Expression

FL-BMDCs are reportedly more steady state-like than GM-BMDCs (42–44). We inquired whether the increased Flt3 expression observed in BEN-exposed FL-BMDCs equated to enhancement of Flt3L-driven steady state features. We assessed B7 molecule expression on FL-BMDCs and found a progressive increase in expression of CD80 (**Figure 2A**) and CD86 (**Figure 2B**) by percent, but not by MFI **(Supplementary Figures 1A, B)**, as the

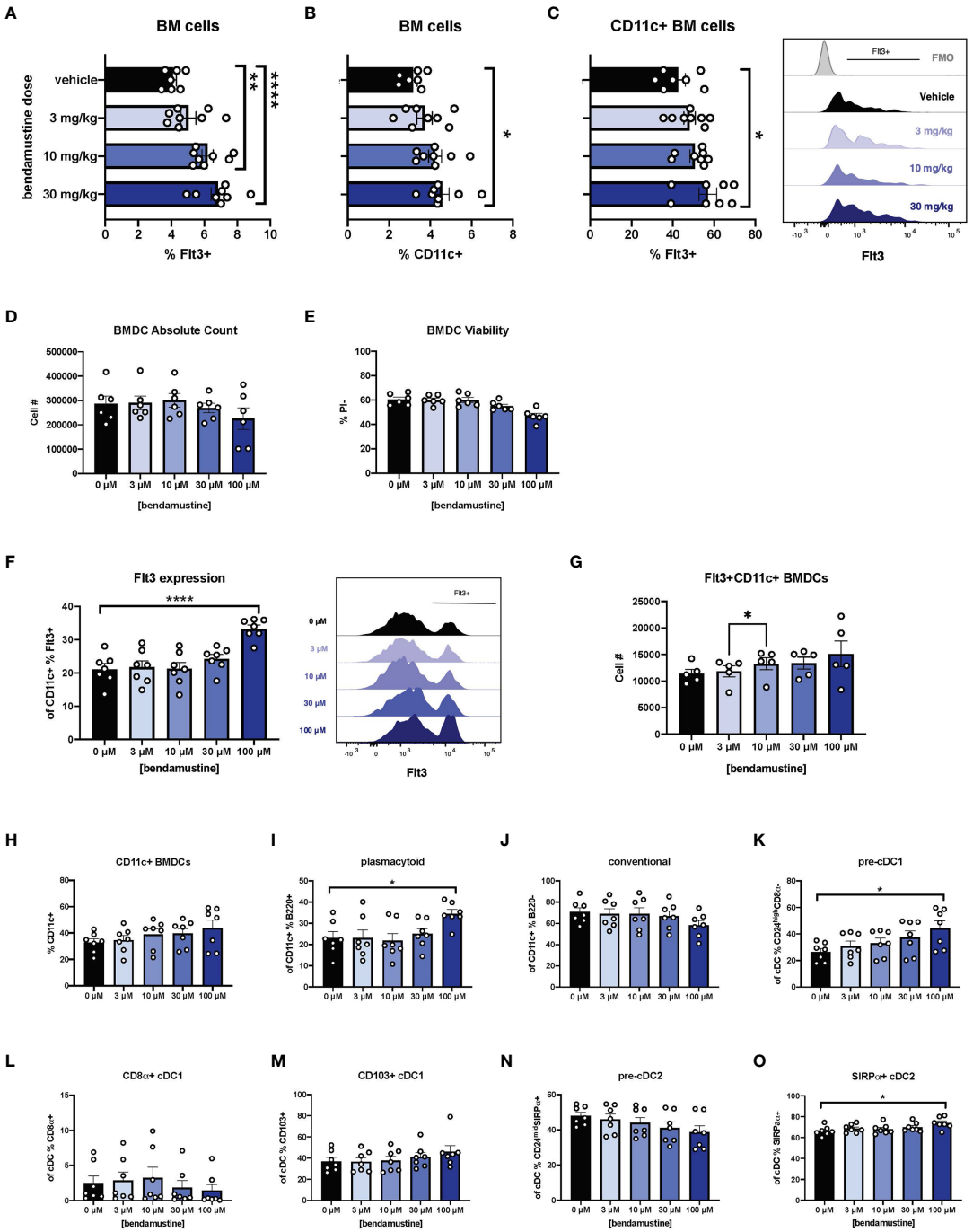


FIGURE 1 | Dose-dependent and concentration-dependent increases in Flt3 expression and skewed DC composition of murine DCs exposed to BEN. **(A–C)** BALB/c mice were i.v. injected with various doses of bendamustine or vehicle and bone marrow (BM) was harvested 48 hours later for analysis by flow cytometry. Data is pooled from 3 independent experiments (n=7-8). **(A)** Mean percent Flt3 expression on total BM cells is shown with SEM. **(B)** The mean percentage of $CD11c^+$ DCs within bone marrow is shown with SEM. **(C)** Among $CD11c^+$ DCs within bone marrow, mean percent Flt3 expression is shown with SEM. Representative histogram shown (right) with Fluorescence Minus Once (FMO) control. **(D–N)** BALB/c FL-BMDCs were generated following brief exposure to BEN and characterized by flow cytometry. Data is pooled from 3 independent experiments (n=6-7). **(D)** Mean absolute cell number and **(E)** percent viable (Propidium Iodide-) cells are shown with SEM. **(F)** Mean percent Flt3 expression among $CD11c^+$ FL-BMDCs is shown with SEM (left) and representative histograms (right). **(G)** Mean absolute cell number of $Flt3^+$ $CD11c^+$ FL-BMDCs is shown with SEM. **(H–N)** Mean percent with SEM of murine DC lineages including **(H)** total $CD11c^+$, **(I)** plasmacytoid DCs ($CD11c^+B220^+$), **(J)** conventional DCs ($CD11c^+B220^-$), **(K)** pre-cDC1s ($CD11c^+B220^-CD24^{high}CD8\alpha^-$), **(L)** $CD8\alpha^+$ cDC1s ($CD11c^+B220^-CD8\alpha^+$), **(M)** $CD103^+$ cDC1s ($CD11c^+B220^-CD103^+$), **(N)** pre-cDC2s ($CD11c^+B220^-$ $CD24^{mid}SIRP\alpha^+$), and **(O)** $SIRP\alpha^+$ cDC2s ($CD11c^+B220^-SIRP\alpha^+$). One-way ANOVA and Dunnett's multiple comparisons test were used to determine significance among groups. *P < 0.05, **P < 0.01, ****P < 0.0001.

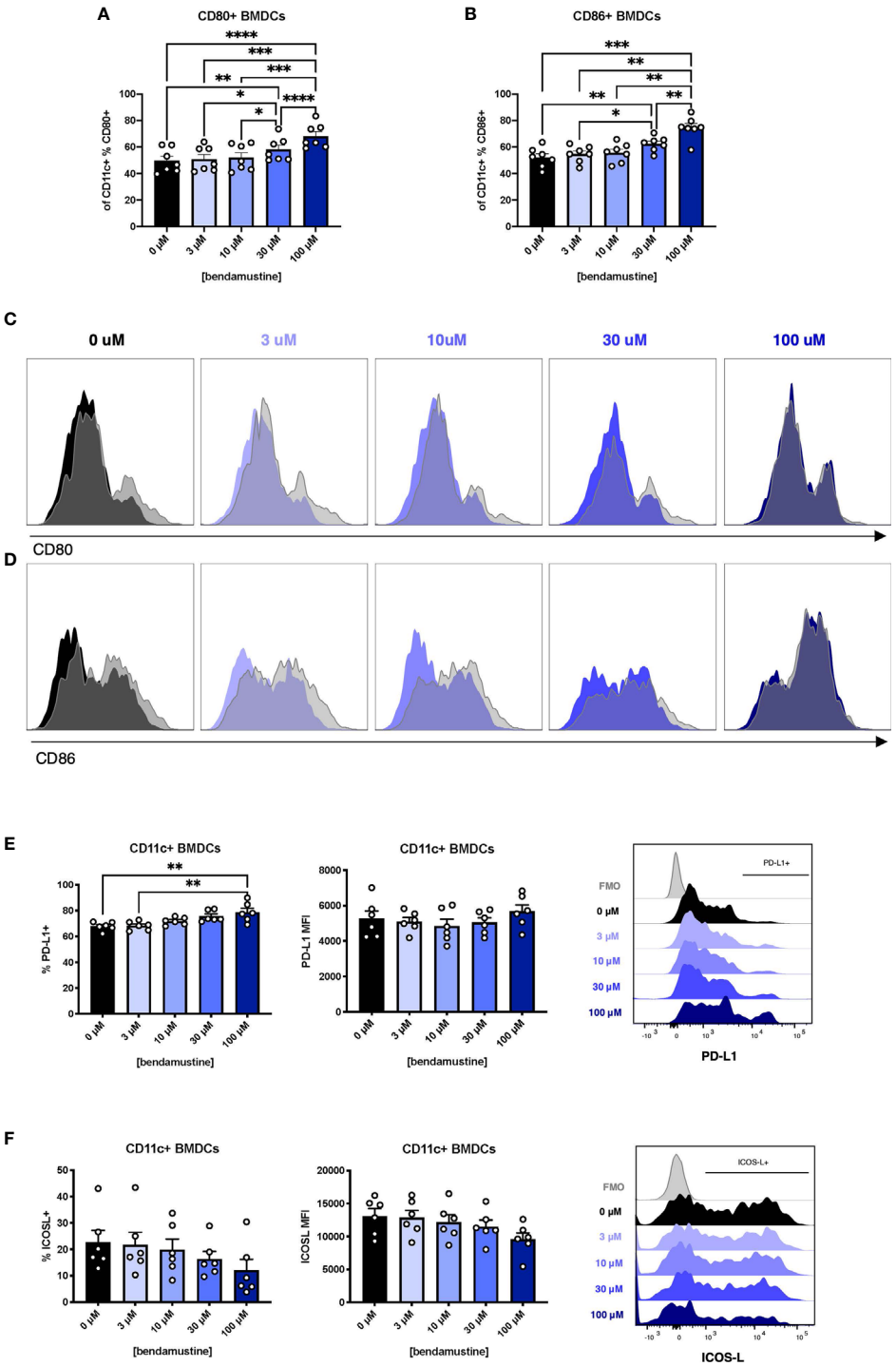


FIGURE 2 | Concentration-dependent increase in CD80, CD86, and PD-L1 expression and dampened response to LPS by murine DCs exposed to BEN. **(A–F)** BALB/c FL-BMDCs were generated following brief exposure to BEN and characterized by flow cytometry. Data is pooled from 3 independent experiments (n=6-7). Mean percent CD80 **(A)** and CD86 **(B)** expression among CD11c$^+$ FL-BMDCs is shown with SEM. Representative histograms of CD80 **(C)** and CD86 **(D)** expression on CD11c$^+$ FL-BMDCs exposed to the indicated concentration of BEN. Unstimulated condition shown in solid color and corresponding LPS stimulated condition overlaid in gray. **(E)** Mean percent PD-L1 expression (left) and MFI (middle) among unstimulated CD11c$^+$ FL-BMDCs shown with SEM, and representative histogram (right). **(F)** Mean percent ICOS-L expression (left) and MFI (middle) among unstimulated CD11c$^+$ FL-BMDCs shown with SEM, and representative histogram (right). One-way ANOVA and Dunnett's multiple comparisons test were used to determine significance among groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

concentration of BEN increases. With 100μM BEN exposure, FL-BMDCs did not exhibit any increase in CD80 or CD86 expression upon LPS stimulation, depicted in histograms with LPS-stimulated DCs overlaid in gray (**Figures 2C, D**) and quantified in **(Supplementary Figures 1C, D)**. Extending our analyses to other co-signaling molecules we demonstrate that the percent expression of PD-L1 significantly increased with higher concentrations of BEN (**Figure 2E**), while the opposite was true with ICOS-L expression (**Figure 2F**). We found no significant changes in expression of CD70, PIR-B, or indoleamine 2,3-dioxygenase **(Supplementary Figures 2A–C)**. All together, we ascertained that BEN-exposed FL-BMDCs are less responsive to LPS stimulation and exhibit greater PD-L1 expression.

BEN Exposure Inhibits Pro-Inflammatory Cytokine Secretion

We next examined pro-inflammatory cytokine secretion by BEN-exposed FL-BMDCs by measuring cytokine concentrations in culture supernatants. Pro-inflammatory cytokines were negligible in unstimulated FL-BMDC cultures. Upon LPS stimulation, control FL-BMDCs (0μM) showed a robust increase in the pro-inflammatory cytokines and chemokines IL-6, TNFα, CCL5, and CCL2 (**Figures 3A–D**). Concentrations of these pro-inflammatory cytokines moderately decreased as the concentration of BEN exposure increased, with a steep drop-off at 100μM (**Figure 3A–D**). However, we did not observe the same effect with the anti-inflammatory cytokine IL-10 (**Figure 3E**). We also found that 100μM BEN exposure significantly hinders secretion of IL-12p40 in response to LPS (**Figure 3F**), though IL-12p70 and IL-23 levels remained very low in all conditions (**Figures 3G, H**). Statistical significance between concentrations of BEN are shown in **(Supplementary Figures 3A–H)**. Intracellular cytokine staining revealed no deficit in intracellular levels of IL-6, TNFα, CCL5, or IL-10, and significantly increased CCL2 (**Figures 3I–M**) in 100μM BEN-exposed FL-BMDCs. These results indicate that BEN exposure diminished secretion of pro-inflammatory cytokines by FL-BMDCs in response to LPS.

BEN-Exposed FL-BMDCs Induce Allogeneic CD4+ T-Cell Proliferation Followed by Cell Death

We next asked whether the changes in co-signaling molecule expression and pro-inflammatory response of BEN-exposed FL-BMDCs affects alloreactive T-cell responses. Enriched live FL-BMDCs were co-cultured with CellTrace-stained allogeneic T-cells in a mixed leukocyte reaction (MLR). Allogeneic T-cells stimulated with BEN-exposed FL-BMDCs exhibited significantly greater allogeneic T-cell proliferation (**Figure 4A**), quantified by proliferation index (**Figure 4B**), on day 3. Most proliferation was among $CD4^+$ T-cells (60-70%), with $CD8^+$ T-cells comprising <5% of proliferated T-cells and the remainder being double negative for CD4 and CD8 **(Supplementary Figures 4A–C)**. We further interrogated the phenotype of the alloreactive T-cells by measuring expression of various markers of T-cell activation, anergy, or exhaustion. 100μM BEN-exposed FL-BMDCs induced greater expression of TIM-3, a marker of T-cell exhaustion, as well as ICOS and CD69, markers of T-cell activation (**Figures 4C–E**). FL-BMDCs previously exposed to 100μM of BEN also induced significantly greater expression of PD-1 (**Figure 4F**). PD-1 is a negative regulator of immune responses and plays a central role in generating peripheral tolerance by promoting programmed cell death of antigen-specific T-cells. We next measured alloreactive T-cell death, which is reportedly induced by Flt3L-expanded DCs (23). As activated T-cells are known to upregulate phosphatidylserine, Annexin V was not used to quantify alloreactive T-cell death. On day 4 of co-culture, we first gated on proliferated, allogeneic ($CellTrace^{low}H2K^{b+}$) T-cells and then quantified T-cell death by PI-positive staining (**Figure 4G**). When we calculate T-cell death as a percentage of all allogeneic T-cells in culture we find that those stimulated with 100μM BEN-exposed FL-BMDCs exhibited significantly greater T-cell death, with 50% of all T-cells dead on day 4 (**Figure 4H**), most of which were $CD4^+$ T-cells (**Figure 4I**). T-cell death induced by 100μM BEN-exposed FL-BMDCs was significantly greater than death observed following stimulation with CD3/CD28 beads **(Supplementary Figure 4D)**, which induced greater T-cell proliferation **(Supplementary Figure 4E)**, indicating that cell death was not merely a result of robust T-cell proliferation. In summary, BEN-exposed FL-BMDCs exhibit an enhanced ability to induce alloreactive T-cell proliferation and cell death.

Previous reports of programmed cell death of alloreactive T-cells have attributed the effect to $CD8\alpha^+$ DCs (23), yet our FL-BMDC system yields fewer than 5% $CD8\alpha^+$ cDC1s (**Figure 1L**). We observe robust frequencies of their immediate precursor, pre-cDC1s (**Figure 1K**), and asked whether pre-cDC1s were maturing into $CD8\alpha^+$ cDC1s in co-culture to induce T-cell death. We demonstrate that by day 3 of co-culture, pre-cDC1s do not mature into $CD8\alpha^+$ cDC1s and remain the predominant population of cDCs (**Figure 4J**). Compared to the FL-BMDC proportions plated on day 0, quantified in **Figures 1H–N**, pre-cDC1s effectively double in percentage, perhaps due to their enhanced life-span compared to $CD8\alpha^+$ cDC1 (47). This indicates that $CD8\alpha^+$ cDC1 may not be the only DC subset capable of inducing deletion of alloreactive T-cells and may signify a previously unknown capability of pre-cDC1s to mitigate alloreactive T-cell responses.

Inhibitor of Flt3 Elicits Similar DC Phenotype

There is a paucity of research on the biological mechanisms of action of BEN. However, one report found that BEN inhibits canonical STAT3 signaling (32). STAT3 is one of several known signaling molecules downstream of Flt3 providing essential signals for differentiation, survival, and proliferation (13, 48–51). We hypothesized that, by inhibiting STAT3, BEN interrupts Flt3-STAT3 signaling causing a compensatory upregulation of Flt3 surface expression. To test this, we performed parallel experiments exposing murine BM cells to pharmacological inhibitors of Flt3 (AC220, Quizartinib) and STAT3 (JSI-124, Cucurbitacin I) for 4 hours, washing, and then generating

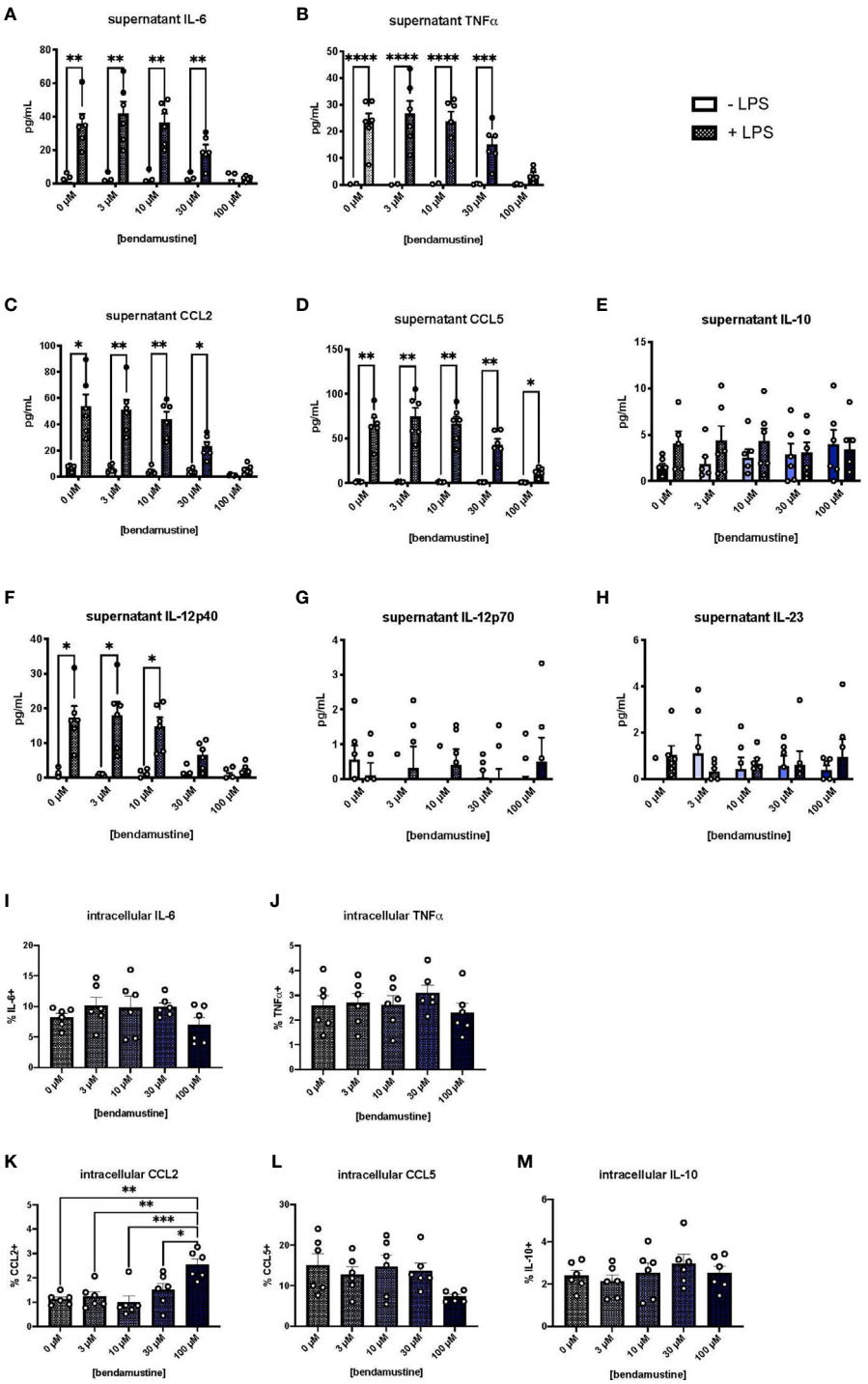


FIGURE 3 | Hindered secretion of pro-inflammatory cytokines in response to LPS by murine DCs exposed to BEN. **(A-H)** BALB/c FL-BMDCs were generated following brief exposure to BEN. With or without 18 hours of LPS stimulation, supernatants were collected for analysis. Data is pooled from 2 independent experiments (n=6). Some values fall below zero, outside of the detectable limits of the assay and outside the axis limits. Mean concentration of **(A)** IL-6, **(B)** TNFα, **(C)** CCL2 (MCP-1), **(D)** CCL5 (RANTES), **(E)** IL-10, **(F)** IL-12p40, **(G)** IL-12p70, and **(H)** IL-23 in supernatants is shown with SEM. Two-way ANOVA and Šidák's multiple comparisons test were used to determine significance among groups. **(I–M)** Murine BMDCs were generated following brief exposure to BEN. BMDCs were stimulated with LPS for 3-4 hours and treated with protein transport inhibitors prior to intracellular cytokine staining protocol. Gating was set based on FMO and isotype controls. Data is pooled from 2 independent experiments (n=6). Mean percent of **(I)** IL-6$^+$, **(J)** TNFα$^+$, **(K)** CCL2$^+$, **(L)** CCL5$^+$, and **(M)** IL-10$^+$ FL-BMDCs shown with SEM. One-way ANOVA and Dunnett's multiple comparisons test were used to determine significance among groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

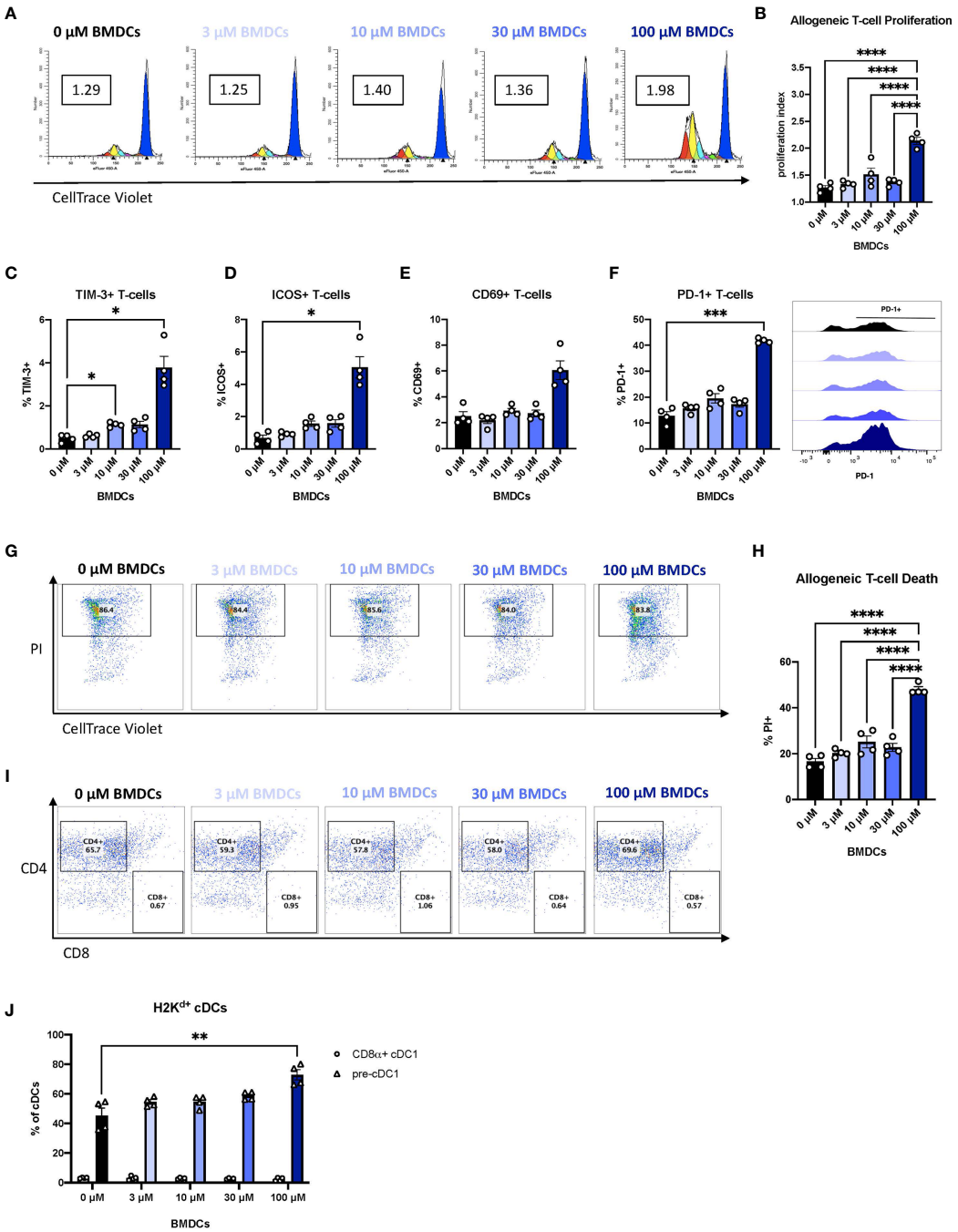


FIGURE 4 | Murine DCs exposed to BEN induce robust proliferation of alloreactive T-cells and expression of PD-1, followed by T-cell death. BALB/c FL-BMDCs were generated following brief exposure to BEN and co-cultured with C57BL/6 CellTrace-stained allogeneic T-cells at a ratio of 1:10. Data shown is representative of 2 independent experiments (n=4). **(A)** Representative histograms generated by ModFit software to quantify T-cell proliferation on day 3 as a proliferation index (PI) (boxed value) in response to stimulation with FL-BMDCs exposed to the indicated concentration of BEN (Gated on $H2K^{b+}$ to exclude DCs from analysis). **(B)** Mean proliferation index on day 3 of co-culture shown with SEM. **(C–E)** Mean percent expression of **(C)** TIM-3, **(D)** ICOS (CD278), and **(E)** CD69 on $H2K^{b+}$ allogeneic T-cells on day 3 shown with SEM. **(F)** Mean percent expression of PD-1 on $H2K^{b+}$ allogeneic T-cells on day 3 shown with SEM, and representative histograms (right). **(G)** Representative flow cytometry plots indicating the percent of dead (PI^+) T-cells within the proliferative fraction (Gated on $H2K^{b+}CellTrace^{low}$). **(H)** Allogeneic T-cell death on day 4 of the assay shown as mean percent of all allogeneic T-cells in culture with SEM. One-way ANOVA and Dunnett's multiple comparisons test were used to determine significance among groups. **(I)** Representative flow cytometry plots indicating the percentages of $CD4^+$ and $CD8^+$ T-cells among dead allogeneic T-cells on day 4 (Gated on $H2K^{b+}CellTrace^{low}PI^+$). **(J)** Mean percent of $CD8\alpha^+$ cDC1s (circles) and pre-cDC1s (triangles) among $H2K^{d+}CD11c^+B220^-$ FL-BMDCs in co-culture with allogeneic T-cells on day 3 shown with SEM. Two-way ANOVA and Šidák's multiple comparisons test were used to determine significance among groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

FL-BMDCs. Similar to our observations with BEN exposure, inhibition of Flt3 prior to FL-BMDC generation results in increased expression of Flt3 (**Figure 5A**), with a less prominent trend resulting from STAT3 inhibition. We observed similar DC composition following Flt3 inhibition with a significant increase in pDCs, pre-cDC1s, and SIRPα^+ cDC2s, and a decrease in pre-cDC2s (**Figures 5B–I**), and a similar trend that was not statistically significant following STAT3 inhibition. We also found similarly increased PD-L1 (**Figure 5J**) and decreased ICOSL expression (**Figure 5K**) following Flt3 inhibition, and to a lesser extent STAT3 inhibition. In support of our hypothesis, exposure to inhibitors of Flt3 and STAT3 phenocopies the effects observed following BEN exposure, with the Flt3 inhibitor showing the most significant response and the STAT3 inhibitor showing slight trends.

Human moDCs Exposed to BEN Have Increased Flt3 Expression and Decreased pSTAT3

Finally, we wanted to determine whether BEN similarly affects human DCs and if so, if those effects are Flt3-STAT3-mediated. We isolated CD14$^+$ monocytes from healthy volunteers to generate moDCs according to established protocols (35–37). Monocytes were exposed to various concentrations of BEN for 4 hours, washed, and moDCs were generated. moDCs exhibited a concentration-dependent increase in Flt3 expression (**Figure 6A**) shown in representative histograms (**Figure 6B**). We also found that these moDCs had significantly decreased phospho-STAT3 (**Figure 6C**). Further studies were conducted to look at DC subsets and found that BEN exposure did not affect moDC purity **(Supplementary Figures 5A, B)** and resulted in a decreased percent of pDCs (**Figure 6D**), a trend toward increased cDC1s (**Figure 6E**), and increased cDC2s (**Figure 6F**). We additionally found small increases in the expression of DNGR1 **(Supplementary Figure 5C)**, another marker for cDC1s, and AXL **(Supplementary Figure 5D)**, a receptor that suppresses inflammatory signaling and limits expression of pro-inflammatory cytokines (52, 53). Consistent with our hypothesis, moDCs exposed to BEN exhibit increased Flt3 expression, decreased pSTAT3, and altered DC composition.

Murine and Human DCs Exhibit Decreased Akt1 Transcripts as the Concentration of BEN Increases

Molecular work to determine whether Flt3-STAT3 signaling is altered was largely inconclusive, with inconsistent changes in transcript levels of Flt3, STAT3, PU.1, Csfr2a, Csf2rb, and Csf3r **(Supplementary Figure 6A)**. Given the sustained inhibition of pSTAT3 in moDCs, we investigated alternative signaling pathways downstream of Flt3 and found that transcript levels of Akt1 were significantly decreased in murine FL-BMDCs **(Supplementary Figure 6B)** and human moDCs **(Supplementary Figure 6C)** exposed to BEN. Protein levels of phosphorylated-Akt1 were largely undetectable in moDC samples making it difficult to make conclusions about the signaling events downstream of Flt3.

DISCUSSION

Graft-versus-host disease remains a significant obstacle to the success of HSCT. Administration of Flt3L *prior* to murine BMT significantly improves GvHD through effects on host DCs (22, 23). Our laboratory has demonstrated that replacing cyclophosphamide with bendamustine, both supplemented with TBI, as a pre-transplant conditioning regimen significantly improves GvHD while maintaining GvL in a murine major-mismatch BMT model (25–27). Extensive investigation of various immune populations following these conditioning regimens found that BEN+TBI conditioning yields donor T-cells that are tolerant to host MHC antigens, yet remain reactive to third-party MHC antigens, while preserving T-cell-dependent GvL (26). We have also reported that BEN+TBI results in robust accumulation of host pre-cDC1s, as well as increased Flt3 expression on host DCs (27). In line with enhanced Flt3 signaling (49), we have reported increased number and suppressive function of myeloid-derived suppressor cells (MDSCs) with BEN+TBI conditioning (25). The biological implications of increased Flt3 expression on DCs are not well understood, and to our knowledge the role of pre-cDC1s in alloreactivity and GvHD has not been previously published.

Our results indicate that BEN increases Flt3 expression in a dose-dependent manner *in vivo* on murine cells, and a concentration-dependent manner *in vitro* in both murine and human cells. We report that BEN-exposure favors the development of murine pDCs, pre-cDC1s, and cDC2s, though further studies would be required to determine whether Flt3 over-expression is responsible for deviations in DC lineage commitment. Increased pDCs and pre-cDC1s were also found in our previous studies with *in vivo* administration of BEN. Of note, concentrations used in our present *in vitro* studies encompass physiological levels reached approximately 2 hours after administration of BEN. While there is no pre-cDC1 equivalent identified in humans, we similarly observed increased cDC2s and a trend toward increased cDC1s, though they differ from murine studies in that we observed a decrease in pDCs. This divergence may be due to the inherent nature of the protocol in that monocytes are exposed to BEN, as opposed to bone marrow cells. We must also note that we did not distinguish monocytic-DCs (Lineage-CD11c$^+$CD16$^+$) in our phenotyping studies. Nevertheless, enhanced Flt3 expression with BEN exposure was consistent between murine and human DCs.

Importantly, administration of Flt3L to the donor does not modify GvHD, and administration of Flt3L to the recipient *post*-transplant accelerates GvHD lethality (21). Further, *in vitro* studies comparing BMDCs generated with Flt3L versus GM-CSF have consistently observed that Flt3L-driven BMDCs are much more steady state-like, producing fewer pro-inflammatory cytokines and inducing less T-cell proliferation (42–44). This body of work suggests that enhancing Flt3 signaling with exogenous Flt3L, specifically among host DCs, results in regulatory DCs that limit alloreactive T-cell responses, are less pro-inflammatory, and prevent GvHD. We posit that this GvHD-suppressing phenotype may extend to our findings with BEN,

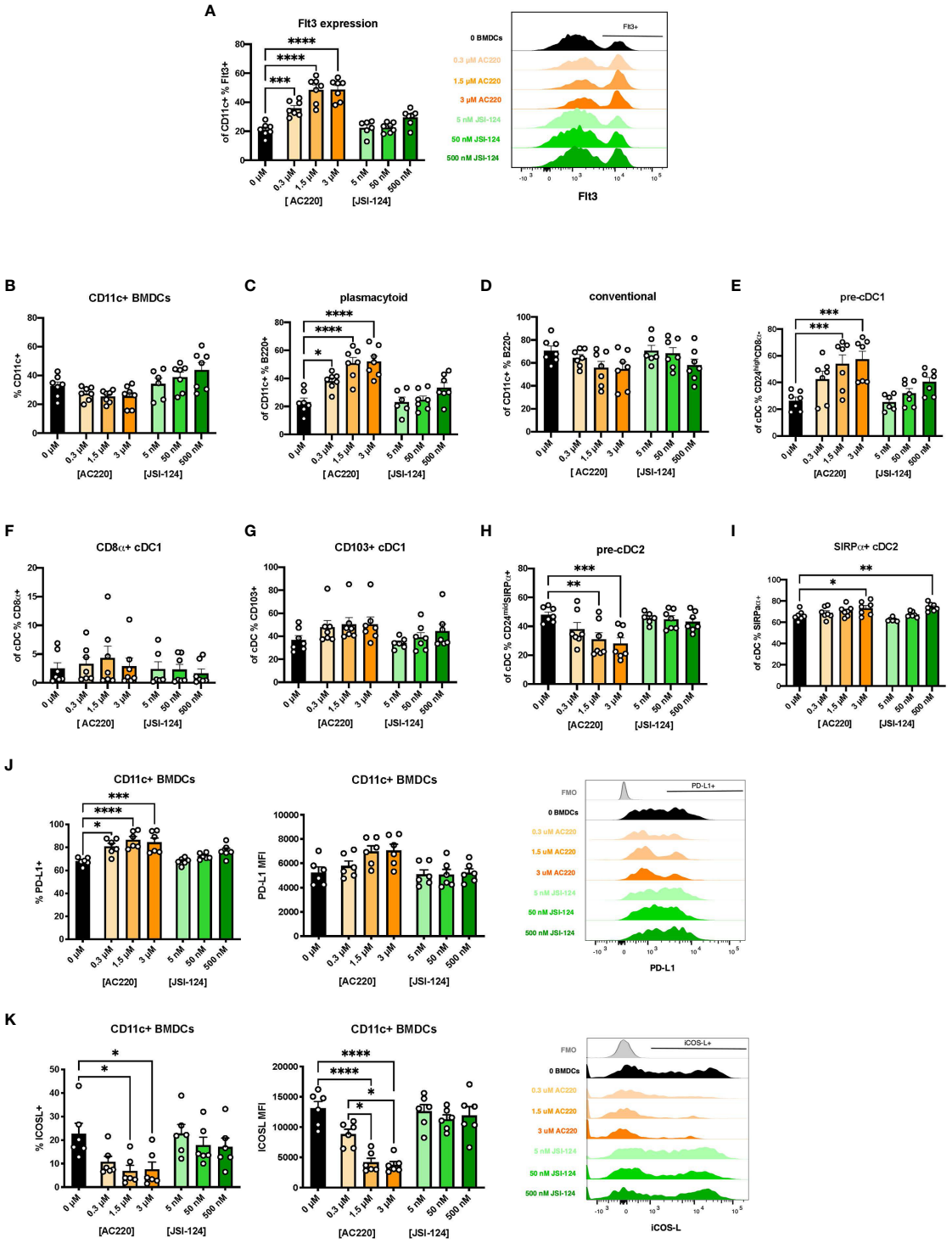


FIGURE 5 | Exposing murine DCs to pharmacological inhibitors of Flt3 and STAT3 phenocopies the effect of BEN exposure. **(A-K)** BALB/c BMDCs were generated following brief exposure to Flt3 inhibitor (ACC20) or STAT3 inhibitor (JSI-124) and characterized by flow cytometry. Data is pooled from 3 independent experiments (n=6-7). **(A)** Mean percent Flt3 expression among $CD11c^+$ BMDCs is shown with SEM, and representative histogram (right). **(B-I)** Mean percent with SEM of murine DC lineages including **(B)** total $CD11c^+$, **(C)** plasmacytoid DCs, **(D)** conventional DCs, **(E)** pre-cDC1s, **(F)** $CD8\alpha^+$ cDC1s, **(G)** $CD103^+$ cDC1s, **(H)** pre-cDC2s, and **(I)** $SIRP\alpha^+$ cDC2s. **(J)** Mean percent PD-L1 expression (left) and MFI (middle) among $CD11c^+$ FL-BMDCs shown with SEM, and representative histograms (right). **(K)** Mean percent ICOS-L expression (left) and MFI (middle) among $CD11c^+$ FL-BMDCs shown with SEM, and representative histograms (right). One-way ANOVA and Dunnett's multiple comparisons test were used to determine significance among groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

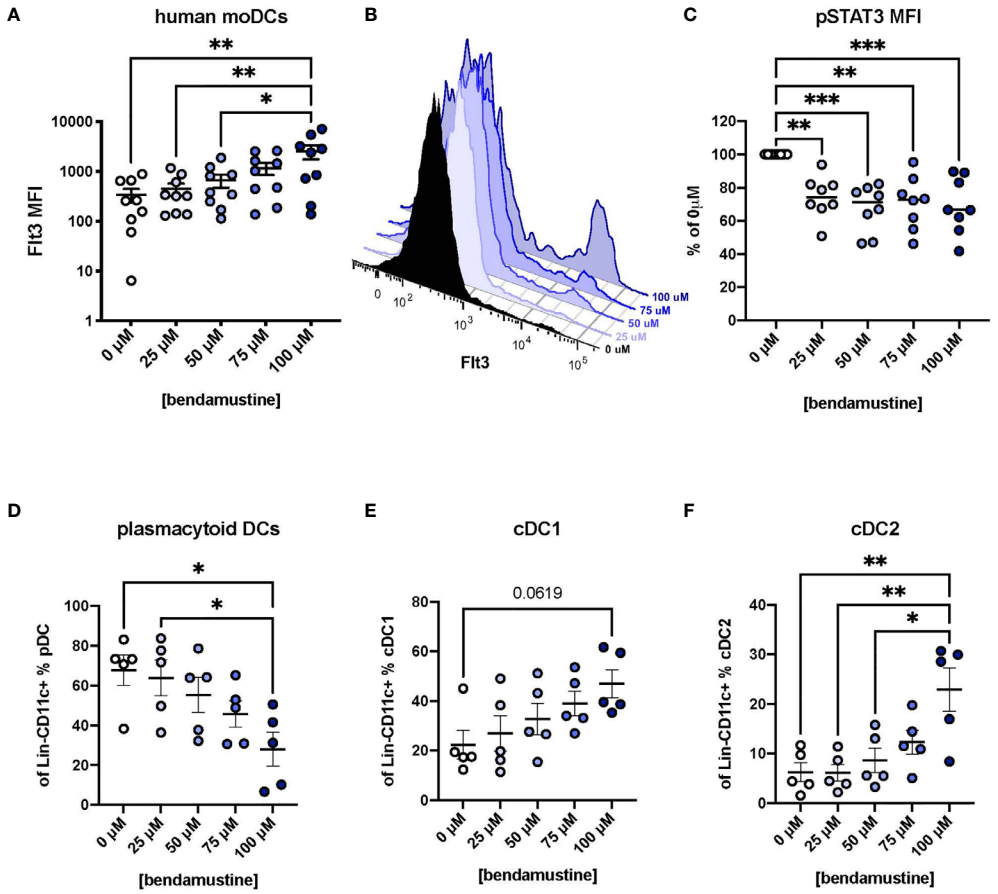


FIGURE 6 | Human DCs exhibit concentration-dependent increase in Flt3 expression following BEN exposure and decreased pSTAT3. Human monocyte-derived DCs (moDCs) were generated following brief exposure to BEN and characterized by flow cytometry. Data shown is pooled from 9 independent experiments (n=5-9). **(A)** Mean Flt3 MFI among human moDCs shown with SEM. **(B)** Representative histogram of Flt3 expression on BEN-exposed moDCs from a single individual. **(C)** Mean pSTAT3 MFI normalized to percent of control (0μM) shown with median. **(D)** Mean percent of plasmacytoid DCs (Lineage$^-$CD11c$^+$BDCA4$^+$) shown with SEM. **(E)** Mean percent of cDC1 (Lineage$^-$CD11c$^+$BDCA4$^-$BDCA3$^+$) shown with SEM. **(F)** Mean percent cDC2 (Lineage$^-$CD11c$^+$BDCA4$^-$BDCA1$^+$) shown with SEM. One-way ANOVA and Dunnett's multiple comparisons test were used to determine significance among groups. *P < 0.05, **P < 0.01, ***P < 0.001.

whereby Flt3 signaling is enhanced *via* increased Flt3 receptor expression on host DCs, rather than with Flt3L administration.

Pre-transplant conditioning regimen components (e.g. total body irradiation) disrupt epithelial barrier integrity and allow translocation of microbial products, such as LPS. GvHD is significantly exacerbated by inflammation caused by recognition of LPS, whereas LPS antagonism has been found to suppress GvHD (54, 55). Using LPS stimulation as a surrogate for total body irradiation experienced *in vivo*, we demonstrate that BEN exposure induces FL-BMDCs that are minimally responsive to LPS. It should be noted that unstimulated FL-BMDCs previously exposed to 100μM of BEN expressed greater percent CD80 and CD86 at baseline, however MFIs were comparable, and upon LPS stimulation exhibited no further increase. FL-BMDCs exposed to 100μM of BEN were found to secrete extremely low levels of pro-inflammatory cytokines and chemokines linked to GvHD development (IL-6, TNFα, CCL2, CCL5, and IL-12p40) (56–59). BEN-exposed BMDCs showed no deficit in IL-10 secretion and no evidence of diminished intracellular levels of these cytokines. This suggests that the phenotype induced by BEN exposure is associated with a suppressed pro-inflammatory response to LPS that may contribute to BEN's protective effect on GvHD.

The outcome of alloreactivity is ultimately determined by the orchestra of co-signaling molecules present during allogeneic T-cell priming (59, 60). We demonstrated a concentration-dependent increase in PD-L1 expression on FL-BMDCs exposed to BEN. PD-L1-mediated inhibitory signaling *via* PD-1 is essential for the induction and maintenance of peripheral tolerance in transplantation (61, 62). T-cells stimulated with 100μM BEN-exposed FL-BMDCs exhibited a striking increase in PD-1 expression and accelerated proliferation, followed by activation-induced death of half of all allogeneic T-cells in culture. The induction of programmed cell death of alloreactive T-cells has been specifically linked to PD-L1 (63) and is critical to induction and maintenance of peripheral tolerance in transplantation (64–67). It is also worth noting that our previous study found

that DCs isolated from BEN-treated mice induced less allogeneic T-cell proliferation compared to CY-treated mice. However, in these previous studies, proliferation was determined by tritiated-thymidine uptake, providing a single snapshot of actively proliferating T-cells. As such, our report of reduced T-cell proliferation from day 3 to day 4 of co-culture may be a reflection of increased alloreactive T-cell death induced by BEN-DCs and is in line with our current findings.

Interestingly, Hill's group has also reported this phenomenon, demonstrating that host $CD8\alpha^+$ cDC1s induce the proliferation and subsequent deletion of allogeneic $CD8^+$ T-cells, and that this effect is enhanced by Flt3L administration (23). We observe deletion of $CD4^+$ T-cells rather than $CD8^+$ T-cells, which is in agreement with Hill's findings since $CD8\alpha^+$ cDC1s constitute a very small proportion of DCs in our assay. This suggests that another Flt3L-driven DC population is capable of inducing specific deletion of alloreactive $CD4^+$ T-cells while sparing $CD8^+$ T-cells, which could potentially preserve GvL responses. We postulate that pre-cDC1s may be responsible for this effect, which may explain why BEN+TBI results in tolerant donor T-cells while maintaining T-cell-dependent, mostly reliant on $CD8^+$ T-cells, GvL (26, 27), though we cannot rule out a contribution of cDC2s.

We found that a Flt3 inhibitor closely replicates many of our findings with BEN, while a STAT3 inhibitor induces similar trends but not significantly so. In agreement with others, we observed decreased levels of phosphorylated Tyrosine 705-STAT3 in human moDCs previously exposed to BEN, though we were surprised that exposure to BEN for just four hours on day 0 resulted in sustained inhibition of STAT3 five days later. Tyrosine 705 is the canonical residue used by Iwamoto's group to determine that BEN binds to and inhibits STAT3, however these studies did not clarify the kinetics of BEN's inhibition of STAT3, nor did they explore other possible post-translational modifications (32). STAT3 is a highly pleiotropic molecule. For instance, STAT3 activation by IL-6 induces phosphorylation of Tyr640, and is required for the suppression of LPS-induced DC maturation (68, 69). Therefore, while BEN inhibits canonical STAT3 signaling *via* phosphorylation of Tyr705, we cannot rule out the possibility that STAT3 may still be activated *via* other post-translational modifications resulting in non-canonical STAT3 activation.

Additionally, Flt3L is sufficient and indispensable for the commitment of progenitors to the committed DC progenitor (CDP) stage of DC development, a commitment step that reportedly requires STAT3 (13). However, others have reported that various Flt3L-mediated DC lineage commitment steps alternatively require PI3K, Akt, and mTORC (9, 48). Activation of Akt1/PI3K/mTOR downstream of Flt3 has been shown to play an essential role in regulating lifespan, pro-inflammatory cytokine production, and autophagy in DCs (33, 48, 51, 70–72). In recent years, the regulation of autophagy in DCs has been shown to affect long-term storage and cross-presentation of antigen and critically determine GvHD and GvL effects (33, 73–75). We found a concentration-dependent decrease in transcript levels of Akt1 in both murine FL-BMDCs and human moDCs. This may indicate that Akt1 transcripts were translated into protein by day 6 of culture, however we were unable to measure protein levels of Akt1 to test this. While our current studies do not clearly define the signaling mechanisms associated with BEN exposure, they suggest differential modulation of the signaling events downstream of Flt3. Additionally, the phenotype we observe here closely resembles that of Flt3L-driven BMDCs, supporting the overarching hypothesis that BEN elicits these effects in DCs by positively modulating the Flt3 signaling pathway.

In summary, we demonstrated that bendamustine directly increases Flt3 expression on murine and human DCs and affects DC ontogeny. BEN-exposure and enhanced Flt3 expression are associated with a distinct semi-mature phenotype in murine FL-BMDCs, with greater CD80 and CD86 expression, but increased PD-L1 expression and dampened cytokine response to LPS stimulation. These regulatory FL-BMDCs induced robust proliferation of alloreactive $CD4^+$ T-cells followed by programmed cell death. This effect may be attributable to pre-cDC1s and appears to spare $CD8^+$ T-cells, providing a potential mechanism by which BEN+TBI conditioning limits GvHD and yields donor T-cells that are tolerant to host antigen while maintaining T-cell-dependent GvL (26).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The University of Arizona's Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The University of Arizona, Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MM designed and performed experiments, analyzed and reviewed data, and wrote the manuscript. EH and JS helped design and perform experiments, reviewed data, and revised the manuscript. NK, KS, FB, and TZ performed experiments and analyzed data. RS contributed to the experimental design, data interpretation and discussion and revised the manuscript. EK supervised and advised on the implementation and conduction of experiments, reviewed and interpreted data, and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This work was supported by Courtney's Courage, PANDA, and Hyundai Hope on Wheels. MM is a PhD Candidate at the

University of Arizona. This work is submitted in partial fulfillment of the requirements for the PhD of MM. The authors wish to thank Vanessa Frisinger for administrative assistance. We'd also like to thank the University of Arizona Flow Cytometry Core Facility for the use of their analytical software and facilities.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.699128/full#supplementary-material

REFERENCES

1. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR. A Receptor Tyrosine Kinase Specific to Hematopoietic Stem and Progenitor Cell-Enriched Populations. *Cell* (1991) 65(7):1143–52. doi: 10.1016/0092-8674(91)90010-V
2. Small D, Levenstein M, Kim E, Carow C, Amin S, Rockwell P, et al. Stk-1, the Human Homolog of Flk-2/Flt-3, Is Selectively Expressed in CD34+ Human Bone Marrow Cells and Is Involved in the Proliferation of Early Progenitor/Stem Cells. *Proc Natl Acad Sci USA* (1994) 91(2):459–63. doi: 10.1073/pnas.91.2.459
3. Kazi JU, Ronnstrand L. FMS-Like Tyrosine Kinase 3/Flt3: From Basic Science to Clinical Implications. *Physiol Rev* (2019) 99(3):1433–66. doi: 10.1152/physrev.00029.2018
4. Rappold I, Ziegler BL, Kohler S, Marchetto O, Rosnet D, Birnbaum D, et al. Functional and Phenotypic Characterization of Cord Blood and Bone Marrow Subsets Expressing FLT3 (CD135) Receptor Tyrosine Kinase. *Blood* (1997) 90(1):111–25. doi: 10.1182/blood.V90.1.111
5. Graddis TJ, Brasel K, Friend D, Srinivasan S, Wee S, Lyman SD, et al. Structure-Function Analysis of FLT3 Ligand-FLT3 Receptor Interactions Using a Rapid Functional Screen. *J Biol Chem* (1998) 273(28):17626–33. doi: 10.1074/jbc.273.28.17626
6. Sitnicka E, Bryder D, Theilgaard-Monch K, Buza-Vidas N, Adolfsson J, Jacobsen SE. Key Role of Flt3 Ligand in Regulation of the Common Lymphoid Progenitor But Not in Maintenance of the Hematopoietic Stem Cell Pool. *Immunity* (2002) 17(4):463–72. doi: 10.1016/S1074-7613(02)00419-3
7. Lyman SD. Biologic Effects and Potential Clinical Applications of Flt3 Ligand. *Curr Opin Hematol* (1998) 5(3):192–6. doi: 10.1097/00062752-199805000-00008
8. Lyman SD, Jacobsen SE. C-Kit Ligand and Flt3 Ligand: Stem/Progenitor Cell Factors With Overlapping Yet Distinct Activities. *Blood* (1998) 91(4):1101–34. doi: 10.1182/blood.V91.4.1101
9. Ceredig R, Rauch M, Balciunaite G, Rolink AG. Increasing Flt3L Availability Alters Composition of a Novel Bone Marrow Lymphoid Progenitor Compartment. *Blood* (2006) 108(4):1216–22. doi: 10.1182/blood-2005-10-006643
10. McKenna HJ, Stocking KL, Miller RE, Brasel K, De Smedt T, Maraskovsky E, et al. Mice Lacking Flt3 Ligand Have Deficient Hematopoiesis Affecting Hematopoietic Progenitor Cells, Dendritic Cells, and Natural Killer Cells. *Blood* (2000) 95(11):3489–97. doi: 10.1182/blood.V95.11.3489.011k45_3489_3497
11. McKenna HJ. Role of Hematopoietic Growth Factors/Flt3 Ligand in Expansion and Regulation of Dendritic Cells. *Curr Opin Hematol* (2001) 8(3):149–54. doi: 10.1097/00062752-200105000-00004
12. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG. Flt3 Ligand Regulates Dendritic Cell Development From Flt3+ Lymphoid and Myeloid-Committed Progenitors to Flt3+ Dendritic Cells *In Vivo*. *J Exp Med* (2003) 198(2):305–13. doi: 10.1084/jem.20030323
13. Laouar Y, Welte T, Fu XY, Flavell RA. STAT3 Is Required for Flt3L-Dependent Dendritic Cell Differentiation. *Immunity* (2003) 19(6):903–12. doi: 10.1016/S1074-7613(03)00332-7
14. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The Origin and Development of Nonlymphoid Tissue CD103+ Dcs. *J Exp Med* (2009) 206(13):3115–30. doi: 10.1084/jem.20091756
15. Saito Y, Boddupalli CS, Borsotti C, Manz MG. Dendritic Cell Homeostasis Is Maintained by Nonhematopoietic and T-Cell-Produced Flt3-Ligand in Steady State and During Immune Responses. *Eur J Immunol* (2013) 43(6):1651–8. doi: 10.1002/eji.201243163
16. Fujita K, Chakarov S, Kobayashi T, Sakamoto K, Voisin B, Duan K, et al. Cell-Autonomous FLT3L Shedding Via ADAM10 Mediates Conventional Dendritic Cell Development in Mouse Spleen. *Proc Natl Acad Sci USA* (2019) 116(29):14714–23. doi: 10.1073/pnas.1818907116
17. Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, et al. Dramatic Increase in the Numbers of Functionally Mature Dendritic Cells in Flt3 Ligand-Treated Mice: Multiple Dendritic Cell Subpopulations Identified. *J Exp Med* (1996) 184(5):1953–62. doi: 10.1084/jem.184.5.1953
18. Stenger EO, Turnquist HR, Mapara MY, Thomson AW. Dendritic Cells and Regulation of Graft-Versus-Host Disease and Graft-Versus-Leukemia Activity. *Blood* (2012) 119(22):5088–103. doi: 10.1182/blood-2011-11-364091
19. Yu H, Tian Y, Wang Y, Mineishi S, Zhang Y. Dendritic Cell Regulation of Graft-Vs.-Host Disease: Immunostimulation and Tolerance. *Front Immunol* (2019) 10:93. doi: 10.3389/fimmu.2019.00093
20. Toubai T, Mathewson N, Reddy P. The Role of Dendritic Cells in Graft-Versus-Tumor Effect. *Front Immunol* (2014) 5:66. doi: 10.3389/fimmu.2014.00066
21. Blazar BR, McKenna HJ, Panoskaltsis-Mortari A, Taylor PA. Flt3 Ligand (FL) Treatment of Murine Donors Does Not Modify Graft-Versus-Host Disease (GVHD) But FL Treatment of Recipients Post-Bone Marrow Transplantation Accelerates GVHD Lethality. *Biol Blood Marrow Transpl* (2001) 7(4):197–207. doi: 10.1053/bbmt.2001.v7.pm11349806
22. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, et al. Flt3 Ligand Therapy for Recipients of Allogeneic Bone Marrow Transplants Expands Host CD8 Alpha(+) Dendritic Cells and Reduces Experimental Acute Graft-Versus-Host Disease. *Blood* (2002) 99(5):1825–32. doi: 10.1182/blood.V99.5.1825
23. Markey KA, Kuns RD, Browne DJ, Gartlan KH, Robb RJ, Martins JP, et al. Flt-3L Expansion of Recipient Cd8alpha(+) Dendritic Cells Deletes Alloreactive Donor T Cells and Represents an Alternative to Posttransplant Cyclophosphamide for the Prevention of GVHD. *Clin Cancer Res* (2018) 24(7):1604–16. doi: 10.1158/1078-0432.CCR-17-2148
24. Stokes J, Hoffman EA, Zeng Y, Larmonier N, Katsanis E. Post-Transplant Bendamustine Reduces GvHD While Preserving GvL in Experimental Haploidentical Bone Marrow Transplantation. *Br J Haematol* (2016) 174(1):102–16. doi: 10.1111/bjh.14034
25. Stokes J, Hoffman EA, Molina MS, Eremija J, Larmonier N, Zeng Y, et al. Bendamustine With Total Body Irradiation Limits Murine Graft-Versus-Host Disease in Part Through Effects on Myeloid-Derived Suppressor Cells. *Biol Blood Marrow Transpl* (2019) 25(3):405–16. doi: 10.1016/j.bbmt.2018.10.009
26. Stokes J, Hoffman EA, Molina MS, Kummet N, Simpson RJ, Zeng Y, et al. Bendamustine With Total Body Irradiation Conditioning Yields Tolerant Cells While Preserving T-Cell-Dependent Graft-Versus-Leukemia. *OncoImmunology* (2020) 9(1):1758011. doi: 10.1080/2162402X.2020.1758011
27. Molina MS, Stokes J, Hoffman EA, Eremija J, Zeng Y, Simpson RJ, et al. Bendamustine Conditioning Skews Murine Host DCs Toward Pre-cDC1s and Reduces Gvhd Independently of Batf3. *Front Immunol* (2020) 11:1410. doi: 10.3389/fimmu.2020.01410
28. Konstantinov SM, Kostovski A, Topashka-Ancheva M, Genova M, Berger MR. Cytotoxic Efficacy of Bendamustine in Human Leukemia and Breast Cancer Cell Lines. *J Cancer Res Clin Oncol* (2002) 128(5):271–8. doi: 10.1007/s00432-002-0331-8
29. Leoni LM, Bailey B, Reifert J, Bendall HH, Zeller RW, Corbeil J, et al. Bendamustine (Treanda) Displays a Distinct Pattern of Cytotoxicity and Unique Mechanistic Features Compared With Other Alkylating Agents. *Clin Cancer Res* (2008) 14(1):309–17. doi: 10.1158/1078-0432.CCR-07-1061
30. Gandhi V, Burger JA. Bendamustine in B-Cell Malignancies: The New 46-Year-Old Kid on the Block. *Clin Cancer Res* (2009) 15(24):7456–61. doi: 10.1158/1078-0432.CCR-08-3041
31. Lu L, Yoshimoto K, Morita A, Kameda H, Takeuchi T. Bendamustine Increases Interleukin-10 Secretion From B Cells Via P38 MAP Kinase

Activation. *Int Immunopharmacol* (2016) 39:273–9. doi: 10.1016/j.intimp.2016.07.033
32. Iwamoto K, Uehara Y, Inoue Y, Taguchi K, Muraoka D, Ogo N, et al. Inhibition of STAT3 by Anticancer Drug Bendamustine. *PloS One* (2017) 12 (1):e0170709. doi: 10.1371/journal.pone.0170709
33. Gao H, Lou S, Hong H, Ge Q, Zhao H. Autophagy Inhibition Potentiates the Anticancer Effects of a Bendamustine Derivative NL-101 in Acute T Lymphocytic Leukemia. *BioMed Res Int* (2020) 2020:1520651. doi: 10.1155/2020/1520651
34. Zeng Y, Stokes J, Hahn S, Hoffman E, Katsanis E. Activated MHC-Mismatched T Helper-1 Lymphocyte Infusion Enhances GvL With Limited Gvhd. *Bone Marrow Transpl* (2014) 49(8):1076–83. doi: 10.1038/bmt.2014.91
35. Dauer M, Obermaier B, Herten J, Haerle C, Pohl K, Rothenfusser S, et al. Mature Dendritic Cells Derived From Human Monocytes Within 48 Hours: A Novel Strategy for Dendritic Cell Differentiation From Blood Precursors. *J Immunol* (2003) 170(8):4069–76. doi: 10.4049/jimmunol.170.8.4069
36. Li G, Zeng Y, Chen X, Larmonier N, Sepassi M, Graner MW, et al. Human Ovarian Tumour-Derived Chaperone-Rich Cell Lysate (CRCL) Elicits T Cell Responses In Vitro. *Clin Exp Immunol* (2007) 148(1):136–45. doi: 10.1111/j.1365-2249.2007.03323.x
37. Kim SJ, Kim G, Kim N, Chu H, Park BC, Yang JS, et al. Human CD141(+) Dendritic Cells Generated From Adult Peripheral Blood Monocytes. *Cytotherapy* (2019) 21(10):1049–63. doi: 10.1016/j.jcyt.2019.07.007
38. Zeng Y, Hahn S, Stokes J, Hoffman EA, Schmelz M, Proytcheva M, et al. Pak2 Regulates Myeloid-Derived Suppressor Cell Development in Mice. *Blood Adv* (2017) 1(22):1923–33. doi: 10.1182/bloodadvances.2017007435
39. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001) 25(4):402–8. doi: 10.1006/meth.2001.1262
40. Angelov GS, Tomkowiak M, Marcais A, Leverrier Y, Marvel J. Flt3 Ligand-Generated Murine Plasmacytoid and Conventional Dendritic Cells Differ in Their Capacity to Prime Naive CD8 T Cells and to Generate Memory Cells *In Vivo*. *J Immunol* (2005) 175(1):189–95. doi: 10.4049/jimmunol.175.1.189
41. Mayer CT, Ghorbani P, Nandan A, Dudek M, Arnold-Schrauf C, Hesse C, et al. Selective and Efficient Generation of Functional Batf3-Dependent CD103+ Dendritic Cells From Mouse Bone Marrow. *Blood* (2014) 124 (20):3081–91. doi: 10.1182/blood-2013-12-545772
42. Weigel BJ, Nath N, Taylor PA, Panoskaltsis-Mortari A, Chen W, Krieg AM, et al. Comparative Analysis of Murine Marrow-Derived Dendritic Cells Generated by Flt3L or GM-CSF/IL-4 and Matured With Immune Stimulatory Agents on the In Vivo Induction of Antileukemia Responses. *Blood* (2002) 100(12):4169–76. doi: 10.1182/blood-2002-04-1063
43. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. Differential Development of Murine Dendritic Cells by GM-CSF Versus Flt3 Ligand Has Implications for Inflammation and Trafficking. *J Immunol* (2007) 179(11):7577–84. doi: 10.4049/jimmunol.179.11.7577
44. Zhang W, Ding Y, Sun L, Hong Q, Sun Y, Han L, et al. Bone Marrow-Derived Inflammatory and Steady State DCs Are Different in Both Functions and Survival. *Cell Immunol* (2018) 331:100–9. doi: 10.1016/j.cellimm.2018.06.001
45. Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, Fuchsberger M, et al. Cutting Edge: Generation of Splenic CD8+ and CD8- Dendritic Cell Equivalents in FMS-Like Tyrosine Kinase 3 Ligand Bone Marrow Cultures. *J Immunol* (2005) 174(11):6592–7. doi: 10.4049/jimmunol.174.11.6592
46. Dubbelman AC, Rosing H, Darwish M, D'Andrea D, Bond M, Hellriegel E, et al. Pharmacokinetics and Excretion of 14C-Bendamustine in Patients With Relapsed or Refractory Malignancy. *Drugs R D* (2013) 13(1):17–28. doi: 10.1007/s40268-012-0001-5
47. Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, et al. Characterization of an Immediate Splenic Precursor of CD8+ Dendritic Cells Capable of Inducing Antiviral T Cell Responses. *J Immunol* (2009) 182 (7):4200–7. doi: 10.4049/jimmunol.0802286
48. Katholnig K, Linke M, Pham H, Hengstschlager M, Weichhart T. Immune Responses of Macrophages and Dendritic Cells Regulated by mTOR Signalling. *Biochem Soc Trans* (2013) 41(4):927–33. doi: 10.1042/BST20130032
49. Rosborough BR, Mathews LR, Matta BM, Liu Q, Raich-Regue D, Thomson AW, et al. Cutting Edge: Flt3 Ligand Mediates STAT3-Independent Expansion But STAT3-Dependent Activation of Myeloid-Derived Suppressor Cells. *J Immunol* (2014) 192(8):3470–3. doi: 10.4049/jimmunol.1300058
50. Shi Y, Zhang Z, Qu X, Zhu X, Zhao L, Wei R, et al. Roles of STAT3 in Leukemia (Review). *Int J Oncol* (2018) 53(1):7–20. doi: 10.3892/ijo.2018.4386
51. Ma W, Liang F, Zhan H, Jiang X, Gao C, Zhang X, et al. Activated FMS-Like Tyrosine Kinase 3 Ameliorates Angiotensin II-Induced Cardiac Remodelling. *Acta Physiol (Oxf)* (2020) 230(2):e13519. doi: 10.1111/apha.13519
52. Zhu C, Wei Y, Wei X. AXL Receptor Tyrosine Kinase as a Promising Anti-Cancer Approach: Functions, Molecular Mechanisms and Clinical Applications. *Mol Cancer* (2019) 18(1):153. doi: 10.1186/s12943-019-1090-3
53. Gay CM, Balaji K, Byers LA. Giving AXL the Axe: Targeting AXL in Human Malignancy. *Br J Cancer* (2017) 116(4):415–23. doi: 10.1038/bjc.2016.428
54. Cooke KR, Gerbitz A, Crawford JM, Teshima T, Hill GR, Tesolin A, et al. LPS Antagonism Reduces Graft-Versus-Host Disease and Preserves Graft-Versus-Leukemia Activity After Experimental Bone Marrow Transplantation. *J Clin Invest* (2001) 107(12):1581–9. doi: 10.1172/JCI12156
55. Noor F, Kaysen A, Wilmes P, Schneider JG. The Gut Microbiota and Hematopoietic Stem Cell Transplantation: Challenges and Potentials. *J Innate Immun* (2019) 11(5):405–15. doi: 10.1159/000492943
56. Cooper AM, Khader SA. Il-12p40: An Inherently Agonistic Cytokine. *Trends Immunol* (2007) 28(1):33–8. doi: 10.1016/j.it.2006.11.002
57. Castor MG, Pinho V, Teixeira MM. The Role of Chemokines in Mediating Graft Versus Host Disease: Opportunities for Novel Therapeutics. *Front Pharmacol* (2012) 3:23. doi: 10.3389/fphar.2012.00023
58. Wu Y, Bastian D, Schutt S, Nguyen H, Fu J, Heinrichs J, et al. Essential Role of Interleukin-12/23p40 in the Development of Graft-Versus-Host Disease in Mice. *Biol Blood Marrow Transpl* (2015) 21(7):1195–204. doi: 10.1016/j.bbmt.2015.03.016
59. Hill GR, Koyama M. Cytokines and Costimulation in Acute Graft-Versus-Host Disease. *Blood* (2020) 136(4):418–28. doi: 10.1182/blood.2019000952
60. Ford ML. T Cell Cosignaling Molecules in Transplantation. *Immunity* (2016) 44(5):1020–33. doi: 10.1016/j.immuni.2016.04.012
61. Tanaka K, Albin MJ, Yuan X, Yamaura K, Habicht A, Murayama T, et al. PDL1 Is Required for Peripheral Transplantation Tolerance and Protection From Chronic Allograft Rejection. *J Immunol* (2007) 179(8):5204–10. doi: 10.4049/jimmunol.179.8.5204
62. Wang L, Han R, Hancock WW. Programmed Cell Death 1 (PD-1) and Its Ligand PD-L1 Are Required for Allograft Tolerance. *Eur J Immunol* (2007) 37 (10):2983–90. doi: 10.1002/eji.200737583
63. Sandner SE, Clarkson MR, Salama AD, Sanchez-Fueyo A, Domenig C, Habicht A, et al. Role of the Programmed Death-1 Pathway in Regulation of Alloimmune Responses In Vivo. *J Immunol* (2005) 174(6):3408–15. doi: 10.4049/jimmunol.174.6.3408
64. Li XC, Wells AD, Strom TB, Turka LA. The Role of T Cell Apoptosis in Transplantation Tolerance. *Curr Opin Immunol* (2000) 12(5):522–7. doi: 10.1016/S0952-7915(00)00133-3
65. Li XC, Strom TB, Turka LA, Wells AD. T Cell Death and Transplantation Tolerance. *Immunity* (2001) 14(4):407–16. doi: 10.1016/S1074-7613(01)00121-2
66. Alpdogan O, van den Brink MR. Immune Tolerance and Transplantation. *Semin Oncol* (2012) 39(6):629–42. doi: 10.1053/j.seminoncol.2012.10.001
67. Robinson KA, Orent W, Madsen JC, Benichou G. Maintaining T Cell Tolerance of Alloantigens: Lessons From Animal Studies. *Am J Transpl* (2018) 18(8):1843–56. doi: 10.1111/ajt.14984
68. Mori R, Wauman J, Icardi L, Van der Heyden J, De Cauwer L, Peelman F, et al. TYK2-Induced Phosphorylation of Y640 Suppresses STAT3 Transcriptional Activity. *Sci Rep* (2017) 7(1):15919. doi: 10.1038/s41598-017-15912-6
69. Park SJ, Nakagawa T, Kitamura H, Atsumi T, Kamon H, Sawa S, et al. IL-6 Regulates In Vivo Dendritic Cell Differentiation Through STAT3 Activation. *J Immunol* (2004) 173(6):3844–54. doi: 10.4049/jimmunol.173.6.3844
70. Park D, Lapteva N, Seethammagari M, Slawin KM, Spencer DM. An Essential Role for Akt1 in Dendritic Cell Function and Tumor Immunotherapy. *Nat Biotechnol* (2006) 24(12):1581–90. doi: 10.1038/nbt1262
71. Hers I, Vincent EE, Tavare JM. Akt Signalling in Health and Disease. *Cell Signal* (2011) 23(10):1515–27. doi: 10.1016/j.cellsig.2011.05.004
72. Yang S, Sun D, Wang L, Wang X, Shi M, Jiang X, et al. The Role of STAT3/mTOR-Regulated Autophagy in Angiotensin II-Induced Senescence of Human Glomerular Mesangial Cells. *Cell Signal* (2019) 53:327–38. doi: 10.1016/j.cellsig.2018.10.021

73. Ho NI, Camps MGM, Verdoes M, Munz C, Ossendorp F. Autophagy Regulates Long-Term Cross-Presentation by Murine Dendritic Cells. *Eur J Immunol* (2020) 51(4):835–47. doi: 10.1002/eji.202048961
74. Hubbard-Lucey VM, Shono Y, Maurer K, West ML, Singer NV, Ziegler CG, et al. Autophagy Gene Atg16L1 Prevents Lethal T Cell Alloreactivity Mediated by Dendritic Cells. *Immunity* (2014) 41(4):579–91. doi: 10.1016/j.immuni.2014.09.011
75. Oravecz-Wilson K, Rossi C, Zajac C, Sun Y, Li L, Decoville T, et al. Atg5-Dependent Autophagy Uncouples T-Cell Proliferative and Effector Functions and Separates Graft-Versus-Host Disease From Graft-Versus-Leukemia. *Cancer Res* (2021) 81(4):1063–75. doi: 10.1158/0008-5472.CAN-20-1346

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 29 July 2021
doi: 10.3389/fimmu.2021.678918

Immunomodulatory Role and Therapeutic Potential of Non-Coding RNAs Mediated by Dendritic Cells in Autoimmune and Immune Tolerance-Related Diseases

Yifeng Liu[†], *Xiaoze Wang*[†], *Fan Yang*, *Yanyi Zheng*, *Tinghong Ye and Li Yang*[*]

Department of Gastroenterology and Hepatology, Sichuan University-University of Oxford Huaxi Joint Centre for Gastrointestinal Cancer, West China Hospital, Sichuan University, Chengdu, China

OPEN ACCESS

Edited by:
Femke Broere,
Utrecht University, Netherlands

Reviewed by:
Uma Sriram,
Temple University, United States
Wenru Su,
Sun Yat-sen University, China

***Correspondence:**
Li Yang
yangli_hx@scu.edu.cn

[†]*These authors have contributed equally to this work and share first authorship*

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *10 March 2021*
Accepted: *15 July 2021*
Published: *29 July 2021*

Citation:
Liu Y, Wang X, Yang F, Zheng Y, Ye T and Yang L (2021) Immunomodulatory Role and Therapeutic Potential of Non-Coding RNAs Mediated by Dendritic Cells in Autoimmune and Immune Tolerance-Related Diseases. Front. Immunol. 12:678918. doi: 10.3389/fimmu.2021.678918

Dendritic cells (DCs) are professional antigen-presenting cells that act as a bridge between innate immunity and adaptive immunity. After activation, DCs differentiate into subtypes with different functions, at which point they upregulate co-stimulatory molecules and produce various cytokines and chemokines. Activated DCs also process antigens for presentation to T cells and regulate the differentiation and function of T cells to modulate the immune state of the body. Non-coding RNAs, RNA transcripts that are unable to encode proteins, not only participate in the pathological mechanisms of autoimmune-related diseases but also regulate the function of immune cells in these diseases. Accumulating evidence suggests that dysregulation of non-coding RNAs contributes to DC differentiation, functions, and so on, consequently producing effects in various autoimmune diseases. In this review, we summarize the main non-coding RNAs (miRNAs, lncRNAs, circRNAs) that regulate DCs in pathological mechanisms and have tremendous potential to give rise to novel therapeutic targets and strategies for multiple autoimmune diseases and immune tolerance-related diseases.

Keywords: autoimmune disease, immune tolerance, dendritic cell, non-coding RNA, ce-RNAs

INTRODUCTION

The first study of dendritic cells (DCs) was published in 1973, when Ralph Steinman and Zan Cohn discovered a small group of cells with unique stellate morphology by microscopic studies of glass-adhering mouse splenocytes (1). In the mononuclear phagocyte system (MPS), some MPS cells retain incompletely degraded antigen and present it to T cells, thus activating T cells (2). These so-called antigen-presenting cells (APCs) initiate a response by activating T cells, which subsequently stimulate antibody production from B cells, thus bridging innate immunity and adaptive immunity (3). DCs serve as a bridge between innate immunity and adaptive immunity, and the discovery of DCs is the result of efforts to understand the cellular initiating factors of the adaptive immune response (2).

Recent research shows that DCs can be classified into major subtypes based on origin and differentiation state. Human DCs are produced through a lymphoid-specific bone marrow haematopoiesis pathway. DC subset differentiation is affected by different specific transcription factors, among which the roles of IRF8 and IRF4 are particularly important (4–7). Under the regulation of these cellular transcription factors, DCs can differentiate into three main subgroups: plasmacytoid DCs (pDCs), type 1 myeloid/conventional DCs (cDC1s) and type 2 myeloid/conventional DCs (cDC2s) (8). In 2019, Brown et al. further classified cDC2s into cDC2A(T-bet$^+$) and cDC2B(T-bet$^-$) by assessing the expression of T-bet, and they are different from proinflammatory and anti-inflammatory phenotypes *in vivo* (9). In addition, increasing evidence has shown that mature DCs can limit effector T cells and promote the differentiation of regulatory T (Treg) cells to promote the formation of immune tolerance in related diseases (10–12).

Researchers have found that genes encode not only functional products such as proteins but also a variety of unique RNAs (13). Despite a lack of protein-coding regions, *Caenorhabditis elegans* was found to carry some RNAs with conserved functions required for cell development (14). Owing to advances in sequencing technologies, researchers have found a large number of various non-coding RNAs. These non-coding RNAs can be divided into several subsets, including microRNAs (miRNAs), circular RNAs (circRNAs), long non-coding RNAs (lncRNAs), tRNA-derived small RNAs (tsRNAs), ribosomal RNAs (rRNAs), and PIWI-interacting RNAs (piRNAs) (14). Some highly conserved RNAs, including miRNAs (15), circRNAs, and lncRNAs, lacking conservation between species (16), account for approximately 60% of the transcriptional output of human cells (17, 18). It is clear that cellular processes and pathways can be regulated though non-coding RNAs in developmental and pathological settings.

Noncoding RNAs play various roles in the regulation of immune cell differentiation and function. Kuiper et al. observed that conditional depletion of Dicer in mouse CD11c+ DCs did not affect the presence of transient resident DCs in lymph nodes or spleen. However, the lack of miRNAs led to a selective loss of these cells in the epidermis, and those cells that did exist lacked the capacity to mature and present antigens (19). Wang et al. demonstrated that lnc-DCs, exclusively expressed in human conventional DCs (cDCs), decreased DC differentiation and reduced the antigen presentation ability of DCs by increasing the expression of STAT3 (20). Zhang et al. found that the expression of circular malat-1 (circ_malat-1) was attenuated by GDF15, leading to repression of the maturation of DCs (21).

Due to the unique role of DCs in immune diseases, researchers have paid more attention to the regulation of DCs by non-coding RNAs in recent years, considering this an important mechanism for further studying the relevant mechanisms and pathological processes in immune diseases. This review summarizes recent developments in non-coding RNA and DC research related to various autoimmune diseases and transplantation immunity, especially highlighting the immunomodulatory role of miRNAs, circRNAs, and lncRNAs in the processes of immune diseases mediated by DCs (**Table 1**).

PLASMACYTOID DENDRITIC CELLS (PDCs)

pDCs are a small subset of DCs that share a similar origin, and pDCs express a narrow range of pattern-recognition receptors (PRRs), including Toll-like receptor 7 (TLR7) and TLR9 (45). Under the stimulation of the above receptors and exogenous or endogenous nucleic acids, pDCs can secrete a large amount of type I IFN and other pro-inflammatory cytokines.

The numbers of pDCs in lymphoid tissues and related target organs, as well as the level of peripheral type I IFN, change in autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and psoriasis (46–48). In SLE, differentiation of Exfo B cells into AFCs requires activation of TRL signalling, which requires the involvement of pDCs (49). Some researchers, therefore, maintain that depletion or functional impairment of pDCs may serve as a viable and potentially specific treatment strategy for lupus (50). In addition to acting directly on autoimmune diseases, pDCs can also affect autoimmunity by regulating other immune cells. Nakamoto et al. demonstrated that bone marrow-derived pDCs induce IL-35 production through Treg cells during ConA-induced acute hepatitis, and the level of type I IFN released by pDCs was also increased. Consequently, the role of pDCs in autoimmune diseases cannot be ignored.

CONVENTIONAL DENDRITIC CELLS (CDCs)

According to the dependence of transcription factors on development, different subtypes of cDC can be divided into

Abbreviations: DCs, dendritic cells; pDCs, plasmacytoid dendritic cells; cDCs, myeloid/conventional dendritic cells; APCs, antigen-presenting cells; MPS, mononuclear phagocyte system; IRF, interferon regulatory factor; Treg, T regulatory cells; STAT3, Signal Transducers and Activators of Transcription 3; GDF15, growth differentiation factor 15; PRRs, pattern-recognition receptors; ConA, Concanavalin A; BDCA, blood dendritic cell antigen; TNF-α, tumour necrosis factor alpha; AIH, autoimmune hepatitis; Tol-DCs, Tolerogenic dendritic cells; MHC, major histocompatibility complex; Mcl-1, myeloid cell leukaemia-1; Bcl-2, B-cell lymphoma-2; TREM-1, Triggering receptor expressed on myeloid cells-1; TGF-β, transforming growth factor beta; MSK1, mitogen-and stress-activated protein kinase 1; NOD2, nucleotide-binding oligomerization domain 2; IBD, Inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; MDP, muramyl dipeptide; pSS, primary Sjogren's syndrome; RA, Rheumatoid arthritis; SLE, Systemic lupus erythematosus; ATP, adenosine triphosphate; IDO, indoleamine 2,3-dioxygenase; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MoDCs, monocyte derived dendritic cells; SSc, Systemic sclerosis; EAM, experimental autoimmune myocarditis; aGVHD, acute graft-versus-host disease; allo-HCT, allogeneic haematopoietic cell transplantation; LPS, Lipopolysaccharide; E2F1, E2F transcription factor 1; ceRNA, competing endogenous RNAs.

TABLE 1 | The targets and regulatory effect of noncoding RNAs on DCs in autoimmune and immune tolerance-related diseases.

Disease	Non-coding RNAs	Type of regulation	DCs (subsets or sources)	Predicted/ identified targets	Function	Refs
SLE	miR574 miR LET7b miR21	↑	pDC	TLR7	Promote pDC maturation and secretion of IFN-α, TNF- and IL-6	(22)
	miR-361-5p, miR-128-3p miR-181a-2-3p	↓	pDC	TLR7	Increase IFN-α secretion	(23)
	miR-155	↑	pDC	TLR7	MHC class II, CD40, CD86 expressions and IFN-α secretion increased	(24)
	miR-29b miR-29c	↓	pDC	TLR9 Mcl-1, Bcl-2	Promote pDCs apoptosis	(25)
	miRNA-150	↓	cDC	TREM-1	inflammation decreased in SLE	(26)
	miR-142-3p	↑	cDC	ND	Increase secretion of related cytokines, inhibit Treg, and promote proliferation of CD4+T	(27)
RA	miR-34a	↑	DCs ($CD1c^+$)	AXL	Promote DCs activation of T cells	(28)
	miR-363	↓	cDC ($CD11C^+av^+$)	ND	Increase Th17 cells differentiation	(29)
pSS	miR-29a mir-29c	↓	pDC	ND	Increase pDCs survival	(30)
	miR-708 miR-130a	↓	cDC ($CD1c^+$)	TLR3, TLR7/8 MSK1	**Increase the secretion of IL-12 and TNF-α**	(31)
IBD	miR-10a	↓	cDC ($CD11c^+$)	IL-12/IL-23p40	Low inflammatory environment in the intestines	(32)
MS	miR-233	↓	cDC ($CD11b^+CD11c^+$)	ND	Inhibit activation of Th17 by decreasing levels of IL-1, IL-6, IL-23	(33)
SSc	miR-31	↑	cDC ($CD11c^+$)	ND	Reduce the number of DC migrations to CNS	(34)
	miR-618	↑	pDC	IRF8	Reduce the development of pDCs in SSc	(35)
Autoimmune myocarditis	miR-223-3p	↑	Tol-DC	NLRP3	Inhibition of DCs maturation	(36)
GVHD	miR-155	↑	DCs (BMDC)	ND	Decrease the migration and inflammatory activation of DC	(37)
	miR-146a	↓	DCs (BMDC, MoDC)	JAK-STAT	Upgrade histopathological GVHD scores	(38)
	miR-29a	↑	DCs (BMDC, MoDC)	TLR7 (mouse) TLR8 (human)	promote DC maturation, migration and activation of T cell proliferation	(39)
SLE	lnc-DC (ENST00000604411.1, ENST00000501122.2)	↑	DCs(MoDC)	ND	Positive correlation with SLEDAI Score	(40)
Autoimmune myocarditis	lncRNA NEAT1	↓	cDCs ($CD80^+$, $CD86^+$, MHC II^+)	Sponge miR-3076-3p NLRP3	Increase DC induced Tregs and inhibited T cells proliferation	(41)
	lncRNA MALAT1	↑	Tol-DCs ($DC\text{-}sign^+$)	mir155-5p	Promote the formation of Tol-DCs	(42)
SLE	circHLA-C	↑	DCs	miR-150	Promote pDCs maturation	(43)
Autoimmune myocarditis	circSnx5	↑	cDC ($CD80^+$, $CD86^+$, MHC II^+)	miR-544	Reduce inflammation of EAM by regulating SOCS1, PU.1	(44)
	circ_Malat-1	↓	cDC ($CD11c^+CD80^+$, $CD86^+$, MHC II^+)	**GDF15 NFκB**	Increase tolerogenic phenotype of DCs	(21)

Increase the secretion of IL-12 and TNF-a; Increased IL-12 and TNF-A secretion in DCs.
GDF15, Growth differentiation factor 15; NF-κB, nuclear factor kappa-B. ND, not done; ↑, upregulated; ↓, downregulated.

cDC1 and cDC2 (51). In the MHC I environment, cDC1s present antigens to immature CD8+ T cells, while in the MHC II environment, cDC2s present more antigens to immature CD4+ T cells (52).

As cells that play a significant role in nonspecific and specific immunity, cDCs are also involved in a variety of autoimmune diseases. The number of cDCs in the peripheral blood of patients with autoimmune diseases (SLE or RA) is related to their localization in the target tissue (53–56). In RA patients, the number of cDCs was found to be increased in synovial fluid and decreased in peripheral blood (57). cDCs appear to express a unique chemokine receptor: CCL6, the CCL20 receptor. CCL20 leads to infiltration of a variety of inflammatory cells, including immature DCs and Th17 effector lymphocytes, and the production of inflammatory cytokines, including TNF-α, IL-1, and IL-17, in inflammatory synovial tissue, which induces recruitment of local cDCs (58, 59). We demonstrated that the role of abnormal autophagy in the immunogenic maturation of cDCs in autoimmune hepatitis should not be ignored, and inhibition of autophagy may be a novel therapeutic strategy for AIH (60).

TOLEROGENIC DENDRITIC CELLS (Tol-DCs)

DCs can promote the tolerance of autoreactive T cells and induce effector T cell differentiation in specific tissue environments, thus affecting autoimmunity, immune tolerance, or both (61). DCs in this state are called tolerogenic DCs (Tol-DCs). However, whether there is a specific sensitized cell origin in the body or whether the sensitized phenotype of DCs reflects their activation state is still unclear (62).

The role of Tol-DCs in autoimmunity is characterized by low expression of costimulatory molecules, production of immunomodulatory cytokines, and inhibition of the proliferation of T cells (63). In addition, the important interaction between Tregs and Tol-DCs in the maintenance of peripheral tolerance in mice and humans cannot be ignored (64). Tol-DCs can promote the differentiation of Treg cells through various mechanisms, such as the production of IL-10, IL-27, TGF and other cytokines and the expression of indoleamine 2,3-dioxygenase (IDO), thereby changing the levels of extracellular adenosine triphosphate (ATP) and adenosine (12, 65–68). Furthermore, treatment centred on tol-DCs administration is yielding promising results as an alternative to immune modulators (69). Tolerant dendritic cells inhibited T cell proliferation and delayed the occurrence of GVHD in mice through lactic acid synthesis (70).

MicroRNAs REGULATE DENDRITIC CELL-MEDIATED AUTOIMMUNE AND IMMUNE TOLERANCE-RELATED DISEASES

Some previous studies have shown that miRNAs can act as regulatory molecules to affect the expression of target genes, thereby altering the immune state of the body (71). MiRNAs influence the pathogenesis of a variety of autoimmune and immune tolerance-related diseases by regulating DCs (**Figure 1**). In terms of treatment, pri-miRNAs may even become innovative drugs for the treatment of immune diseases (72).

Systemic Lupus Erythematosus (SLE)

The cause of SLE is multifactorial, including the environment, random factors and genetic susceptibility (73). Large amounts of type I IFN and various cytokines produced by pDCs are typically found to be statistically related to the aetiopathogenesis of SLE (74). Salvi et al. purified exosomes from plasma collected from SLE patients and extracted miRNAs (idiopathic inflammatory myopathy (IIM) miRNAs: miR574, LET7b, and miR21) that could induce the production of type I IFNs in human pDCs from these exosomes. These miRNAs can act as survival factors for human pDCs, activate the maturation of pDCs, increase the expression of CD86 and decrease BDCA-2 levels as well as the production of IFN and pro-inflammatory cytokines (TNF-α, IL-6) and phosphorylated p65 (a subunit of NF-κB). Moreover, IIM miRNAs represent potential endogenous ligands of human TLR7, which is the specific endosomal single-stranded RNA (ssRNA) receptor expressed by pDCs (22). Hoogen et al.

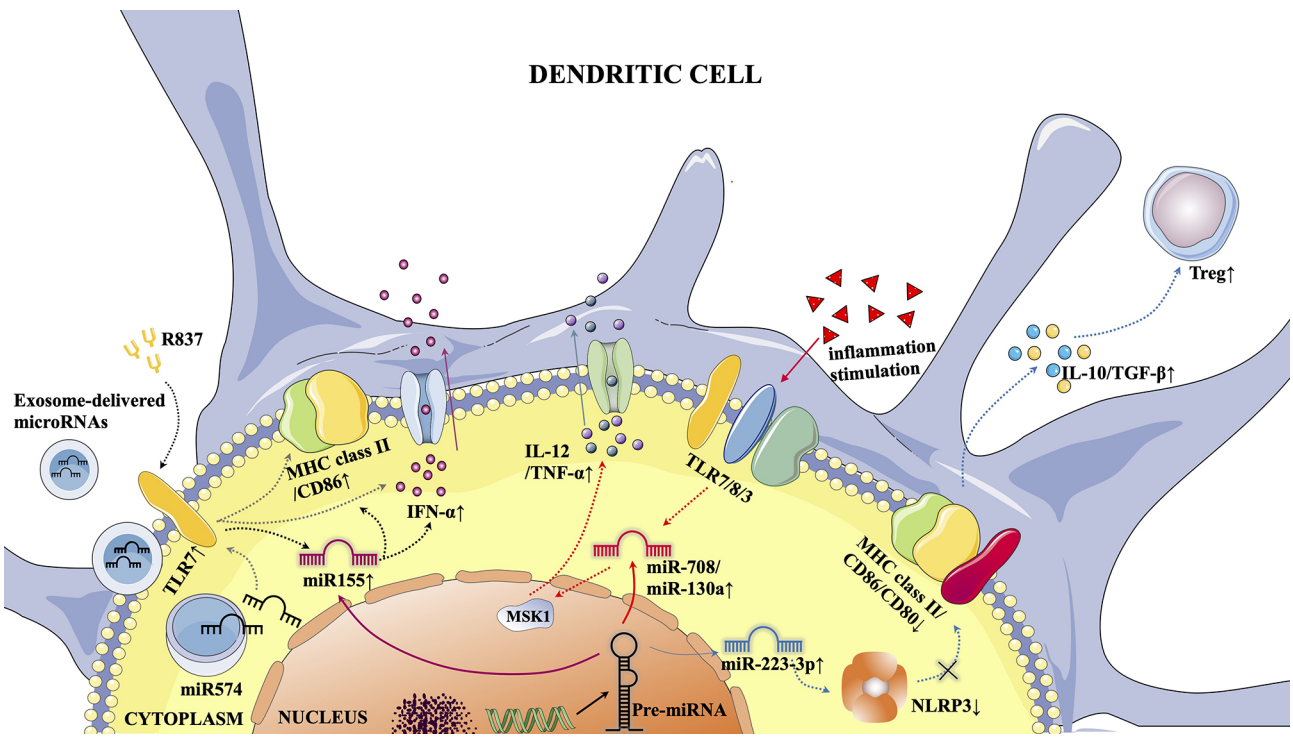


FIGURE 1 | Typical microRNA-mediated pathways in DCs. 1) Activation of TLR7 by the TLR7 agonist R837 resulted in increased miR155 expression, which in turn promoted pDC maturation (elevated MHC class II/CD86 expression) and increased IFN-α secretion. 2) Under the stimulation of extraneous inflammatory factors, TLR7/8/3 was activated, which increased the expression of miR-708/miR-130a, leading to the inhibition of MSK1 and promoting the secretion of IL-12/TNF-α in cDCs. 3) The increased expression of miR-223-3p was followed by inhibition of NLRP3 inflammocytes, thereby promoting the DC tolerance phenotype (decreased EXPRESSION of MHC Class II/CD86/CD80), leading to increased secretion of IL-10/TGF-β and promoting Treg proliferation. 4) Activation of TLR7 by exosome-derived microRNAs through cell membranes can promote pDC maturation and increase IFN-α secretion.

analysed 131 miRNAs in pDCs in SLE and related diseases (SLE + antiphospholipid syndrome and primary antiphospholipid syndrome) and found that 73 of them showed reduced expression. Of the 73 miRNAs, miR-361-5p, miR-128-3p and miR-181a-2-3p were expressed at lower levels in patients with a high IFN signature than in patients with a low IFN signature and healthy controls (23). By employing pDCs from murine models of lupus, Tam et al. discovered that the upregulation of miR-155 was the strongest, and the upregulation of miR-155 was significantly higher in active pDCs from the symptomatic group than in those from the control group. In agreement with this, TLR7-mediated miR-155 overexpression has been shown to lead to elevated CD40 expression (24). This finding is consistent with another study showing that MHC class II, CD40, and CD86 expression is decreased by miR-155 knockdown in Kupffer cells (75). pDCs activated by the TLR pathway are resistant to glucocorticoid-induced apoptosis, which makes glucocorticoids ineffective in the treatment of type I IFN-related autoimmune diseases. In another study, miR-29b and miR-29c promoted pDC apoptosis by directly targeting Mcl-1 and Bcl-2, which elevated the therapeutic effect of glucocorticoids in SLE (25). TLR and IFN receptors are innate immune receptors, and dysregulation of TLR and IFN signalling can lead to innate immune system disorders; these pathways have been shown to be important in lupus pathogenesis (76). As we have previously described, dysregulated miRNAs influence the progression of SLE by regulating pDCs activated by TLRs and/or IFN, as well as by inducing the secretion of inflammatory cytokines.

Not only pDCs but also active cDCs play important roles in the development of SLE. Triggering receptor expressed on myeloid cells-1 (TREM-1) might play a part in the pathogenesis of autoimmune disorders such as lupus through TLR-induced inflammatory responses (77). By selecting and analysing splenocytes from MRL/lpr mice, Gao et al. found that the expression of miR-150 could downregulate the levels of TREM-1, suggesting that TREM-1 may be a therapeutic target for the prevention of inflammatory cDC effects in SLE (26). In addition, miR-142-3p promoted monocyte-derived DCs (moDCs) to secrete CCL2, CCL5, CXCL8, IL-6, TNF-α and other SLE-related cytokines. Moreover, overexpression of miR-142-3p in moDCs inhibited the proliferation of $CD4^+CD25^+Foxp3^+$ Treg cells and recruited more $CD4^+$ T cells, which impacted moDC-CD4+ T cell interactions (27). Regarding Tol-DCs, although a recent publication detailing that adoptive transfer of drug-induced Tol-DC1s and Tol-DC3s reported beneficial therapeutic effects in MRL-Fas^{lpr} lupus-prone mice (78), to date, there have been no relevant studies on the role of miRNAs in regulating DC tolerance in SLE.

Rheumatoid Arthritis (RA)

RA is a chronic and inflammatory synovitis systemic autoimmune disease and is the most frequent autoimmune polyarthritis, with a lifetime prevalence of 3.6% in women and 1.7% in men (79, 80). Activation of DCs is involved in the pathogenesis of RA. Synovial fluid can contain both conventional CD1c+ and inflammatory CD1c+ cells, and these cells not only prime naive T cells (81) but also stimulate TLR7/8 ligands; in response, cytokines such as TNF are produced, thereby promoting synovial inflammation (82). Changes in the expression level of miRNAs can affect the abundance of DC surface receptors and thus regulate the maturation of DCs to change the inflammatory state in RA. A study found that CD1c+ DCs continuously expressed high levels of miR-34a, which inhibited the expression of cellular AXL, a tyrosine kinase receptor, thus contributing to the development of experimental arthritis. This expression of miR-34a may shift DCs towards a mature state, and mature DCs can support autoreactive T cells. Furthermore, in animal studies, compared with wild-type (WT) mice, miR-$34a^{-/-}$ mice had a significantly lower incidence and severity of arthritis (28), which means that miR-34a inhibitors could be a potential treatment for RA. In addition, miRNAs can also affect helper T cell differentiation by regulating DCs, thus affecting the development of RA. Another study found that $CD11C^+av^+$ DCs induced Th17 cell differentiation. A possible mechanism has been proposed: decreased miR-363 expression in DCs from RA patients was shown to upregulate the expression of integrin av, which induced the activation of TGF-β and promoted the differentiation of Th17 cells (29); Th17 cells can exacerbate RA and are directly involved in cartilage and bone destruction (83).

Sjögren's Syndrome

Primary Sjogren's syndrome (pSS) is an autoimmune disease characterized by inflammatory cells infiltrating multiple exocrine glands, such as salivary glands and lacrimal glands, and leads to a series of pathological manifestations, such as sicca keratoconjunctivitis and xerostomia (84). The number of pDCs in the peripheral blood of pSS patients is decreased (85), but in the target organ and salivary glands, the quantity of IFN-α-producing cells is increased (86, 87). Importantly, pDCs can also be activated by endogenous nucleic acids (88). Therefore, pDCs are considered to be the main contributor to the production of type I IFN in pSS and a key mediator of immunopathology. In addition, in pSS, multiple studies have shown that miRNAs are abnormally expressed in multiple tissues and cells of the human body, including purified immune cells, peripheral blood mononuclear cells (PBMCs) and salivary gland tissues (89, 90). In recent years, researchers have also noted the regulatory effects of miRNAs on DCs in pSS. Hillen et al. focused on 20 miRNAs that were differentially expressed between pDCs from patients with pSS and normal controls by an OpenArray quantitative PCR-based technique. In this study, abnormal regulation of the miRNome affected the type I IFN secretion and death of pDC from patients with pSS, and downregulation of pro-apoptotic factors such as miR-29a and miR-29c strengthened the survival of pDCs (30). Not only pDCs but also cDCs are involved in the pathological processes of pSS. cDC2s, which characteristically express CD1c, are the predominant cDCs in human blood, tissues, and lymphatic organs (8). Importantly, CD4+ T cells, the main target cells of cDC2s, play a crucial role in pSS immunopathology (91, 92). Ana P. Lopes et al. found that miR-708 and miR-130a expression in pSS cDC2s was downregulated after activation of some TLRs (TLR3 and TLR7/8), and this altered expression was involved in the

pathogenesis of pSS. In addition, the secretion of inflammatory cytokines was increased. These results suggest that decreased expression of miR-130a and miR-708 can reflect cDC2 activation (31). Furthermore, miR-130a regulates the expression of MSK1, a targeted signalling protein overexpressed in cDC2s in pSS and an upstream mediator of NF-κB that regulates the secretion of some pro-inflammatory cytokines by cDC2s (31, 93).

Inflammatory Bowel Disease (IBD)

A large number of microorganisms accumulate in the intestinal mucosa shortly after birth (94). Studies have shown that in the process of innate immune activation, specific miRNAs are upregulated, thereby affecting the innate response to microbial and viral infections (95). Mature DCs become highly specialized APCs when they encounter microbial products and inflammatory stimulation. Previous research has shown that lamina propria DCs may be associated with specific immune functions in the lamina propria and Peyer plaques (96). Therefore, miRNA-based regulation of DCs in intestinal immunity has gradually become a research focus. In one study, owing to the effects of enteric microorganisms, the expression of the miR-10a precursor was inhibited, which caused decreased expression of IL-12/IL-23p40 in DCs. In line with this finding, a miR-10a inhibitor promoted the expression of IL-12/IL-23p40. The gene encoding IL-12/IL-23p40, IL-12B, has been closely related to susceptibility to Crohn's disease (CD) and somewhat related to susceptibility to ulcerative colitis (UC) (97–99). Another study determined whether abnormal expression of miR-10a in human DCs could inhibit the expression of NOD2, which is a prototypical member of the IL-12/IL-23P40 and nod-like receptor family. Furthermore, NOD2 can be activated by muramyl dipeptide (MDP) from bacteria (32, 100). Researchers have long believed that the NOD2 polymorphism is related to susceptibility to CD (101). Therefore, the regulation of DCs by miR-10a may also be one of the pathological mechanisms underlying IBD.

Multiple Sclerosis

Multiple sclerosis is an autoimmune disease characterized by inflammatory demyelination of white matter in the central nervous system (CNS). The most commonly involved areas are the alba around the ventricle, optic nerve, spinal cord, brainstem and cerebellum. Through analyses of experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS) mouse models, researchers have found that MoDCs, which are $Ly6c^{hi}CD11b^{+}CD11c^{+}$, are important CNS-infiltrating cells (102, 103). Another publication reported that miR-223, which is among the upregulated miRNAs in MS patients (104), plays an important role in inflammation in the CNS by controlling the level of MoDC-secreted Th17-polarizing cytokines (including IL-1β, IL-6 and IL-23) to regulate the induction of the Th17 response (33). Hoye et al. focused on the elevated expression of miR-31 in DCs that migrate through the blood-brain barrier *in vitro*. These results suggest that miR-31 may have potential regulatory effects on DC migration in the CNS during EAE (34). In addition, a recent publication found that miPEP155 can regulate the antigen-presenting capacity of dendritic cells in an inflammatory environment and has a good therapeutic effect on two autoimmune diseases in mouse models of psoriasis and multiple sclerosis (72).

Systemic Sclerosis (SSc)

Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrosis, vascular lesions, and immune dysfunction. pDCs infiltrate the skin of SSc patients and become chronically activated, leading to the secretion of IFN-α and CXCL4, which is characteristic of the disease (105). One publication noted that overexpression of miR-618 reduced the development of pDCs *in vitro* and enhanced the ability of cells to secrete IFN-α, suggesting that miR-618 may be an important epigenetic target for regulating immune system homeostasis in diseases characterized by a type I IFN signature (35).

Autoimmune Myocarditis

As the main cause of sudden death and dilated cardiomyopathy in children and young adults, autoimmune myocarditis features aseptic inflammation of cardiac tissues, and miRNAs play a regulatory role in its induction by inducing the generation of Tol-DCs. A large number of animal models have proven that Tol-DCs can inhibit the occurrence and/or progression of autoimmune diseases through adoptive transfer of BMDCs into mouse models (106–108). A recent study found that the inflammation of heart tissue and poor heart function in experimental autoimmune myocarditis (EAM) mice were reversed after transfusion of miR-223-3p-overexpressing DCs, indicating that miR-223-3p is involved in inducing Tol-DCs and regulating tolerance in autoimmune myocarditis (36).

Acute Graft-*Versus*-Host Disease (aGVHD)

Among immune tolerance-related diseases, acute graft-*versus*-host disease (aGVHD) is a major immune complication that occurs after allogeneic haematopoietic cell transplantation (allo-HCT) due to a series of cytokine storms initiated by the recipient (109). MiRNAs are small non-coding RNAs, and their role in regulating inflammation and innate and adaptive immune responses cannot be ignored. The expression of multiple target mRNAs can be regulated by the same miRNA (110). In recent years, some publications have focused on the crucial role of miRNA dysregulation in DCs in the GVHD pathomechanism. One study noted that miR-155 expression was increased in activated DCs, and the severity of GVHD in miR-$155^{-/-}$ transplant recipients was decreased when DC migration and the level of inflammasome activation were attenuated (37). Stickel et al. revealed that miR-146a can negatively regulate the JAK-STAT signalling pathway in DCs, suggesting that miR-146a variants can significantly increase the risk of acute severe GVHD in human allo-HCT recipients (38). Another study identified a partial role of miR-29a in stimulating DCs through TLR7 and TLR8 (in mice and humans, respectively) to release pro-inflammatory cytokines TNF and IL-6, which are critical drivers of acute GVHD pathogenesis, and to increase T cell proliferation (39). These studies provide a new research paradigm for identifying more effective prevention and treatment strategies for acute GVHD.

LncRNAs REGULATE DENDRITIC CELL-MEDIATED AUTOIMMUNE AND IMMUNE TOLERANCE-RELATED DISEASES

LncRNAs, with lengths over 200 nt, are a group of non-coding RNAs with structures similar to mRNAs but lack any significant open reading frames (111, 112). In addition, they play crucial roles in various biological processes, such as immune cell differentiation, apoptosis and immune responses (20, 113). Many lncRNAs can be induced by TLRs. For example, stimulation of TLR4 induces the expression of lincRNA-Cox2 in CD11C+ BMDCs (15). In the following sections, we summarize previous studies of lncRNAs affecting DCs in autoimmune diseases and transplantation immunity.

Systemic Lupus Erythematosus (SLE)

LncRNAs may be involved in the molecular regulatory mechanisms in lupus (114). Li et al. focused on the expression of lnc-DC in SLE patients, which was significantly lower than that in healthy controls. In contrast, the lnc-DC level was higher in the lupus nephritis group than in the healthy control group. To identify the correlation between differentially expressed lncRNAs in MoDCs of SLE patients and the SLEDAI score, Wang et al. used lncRNA microarrays and qPCR and found that the expression levels of ENST00000604411.1 and ENST00000501122.2 were able to estimate the activity of SLE. Specifically, the expression of these two markers was positively correlated with the SLEDAI score (40). These results suggest that lnc-DC could be a new biomarker for SLE.

Immune Tolerance

In transplantation immunity, abnormal lncRNA expression levels can affect the transformation of DCs into Tol-DCs. Yu et al. confirmed that the expression of the lncRNA NEAT1 was increased in mature DCs induced by LPS. As a ceRNA, NEAT1 regulated NLRP3 expression by affecting the activity of miR-3076-3P, and the expression of lncRNA NEAT1 could be regulated although E2F1 activity mediated by miR Let-7i (**Figure 2**). Thus, transfusion of NEAT1-knockdown DCs into mouse models with EAM and heart transplantation reduced inflammatory cell infiltration, inhibited T cell proliferation, and increased the number of Treg cells (41). Another publication noted that the functional lncRNA MALAT1 is involved in Tol-DC induction and regulation of immune tolerance in heart transplantation and EAM. MALAT1 regulates the formation of Tol-DCs and immune tolerance by functioning as a miR155 sponge in the cytoplasm to promote DC-SIGN and IL10 production (42).

CIRCULAR RNAs REGULATE DENDRITIC CELL-MEDIATED AUTOIMMUNE AND IMMUNE TOLERANCE-RELATED DISEASES

Circular RNAs are widely found in human and mouse genomes, so they are likely to be a common feature of eukaryotic gene expression and regulation, although they were previously ignored (115). In addition, they have been subsequently found in the genes of other animals, including flies and worms, by microarray analysis (116, 117). There is mounting evidence that circRNAs play an essential role in complex human pathologies. circRNAs have been used in some studies as new noninvasive biomarkers for certain autoimmune diseases (118). DCs are regarded as an important class of APCs in autoimmunity. DCs have been found to be involved in various autoimmune diseases and immune tolerance-related diseases; therefore, an in-depth study of the regulatory mechanisms by which circRNAs affect DCs will not only improve our understanding of the molecular mechanisms of these diseases but also make it possible to identify future treatments for them.

Systemic Lupus Erythematosus (SLE)

Recent studies have suggested that circRNAs may play a regulatory role in SLE by serving as miRNA sponges (119, 120) and can be used as potential biomarkers for SLE (120). Another study confirmed that the circRNA hsa_circ_0045272 negatively regulates apoptosis and interleukin-2 secretion in SLE. There are other relevant studies on the regulation of DCs. For example, circHLA-C was shown to play a potentially important role in the pathogenesis of lupus nephritis by sponging miR-150. In addition, through GO analysis, it was found that upregulated circRNAs are involved in regulating the differentiation of DCs and other biological functions (43).

Immune Tolerance

A large number of studies have shown that circRNAs play an important role in the immune system (121), and some circRNAs have been found to be abnormally expressed in DCs with different functions (21). The role of circRNAs in inducing Tol-DCs cannot be ignored. A recent publication found that circSnx5 could bind with miR-544 as a molecular sponge by analysing circSnX5-associated competing endogenous RNA (ceRNA) networks to weaken the inflammatory phenotype of DCs and enhance their tolerance in a heart transplantation mouse model (44) (**Figure 2**). In addition, some upstream regulatory factors may affect the expression of circRNAs to regulate the function of DCs. Another study studied growth differentiation factor 15 (GDF15)-induced Tol-DCs by inhibiting the circ_Malat-1 and NFκB signalling pathways (21). This study indirectly confirmed that the circRNA Malat-1 has a regulatory effect on DCs in immune tolerance.

THE THERAPEUTIC POTENTIAL OF NONCODING RNAs IN AUTOIMMUNE DISEASES

Changing the expression level of non-coding RNAs can further affect the process of autoimmune diseases through the regulation of DC function. As described above, the inflammatory response in SLE can be reduced by reducing the expression of miR-142-3p and miR-150 (27, 77). In addition, miR-29b and miR-29c can also

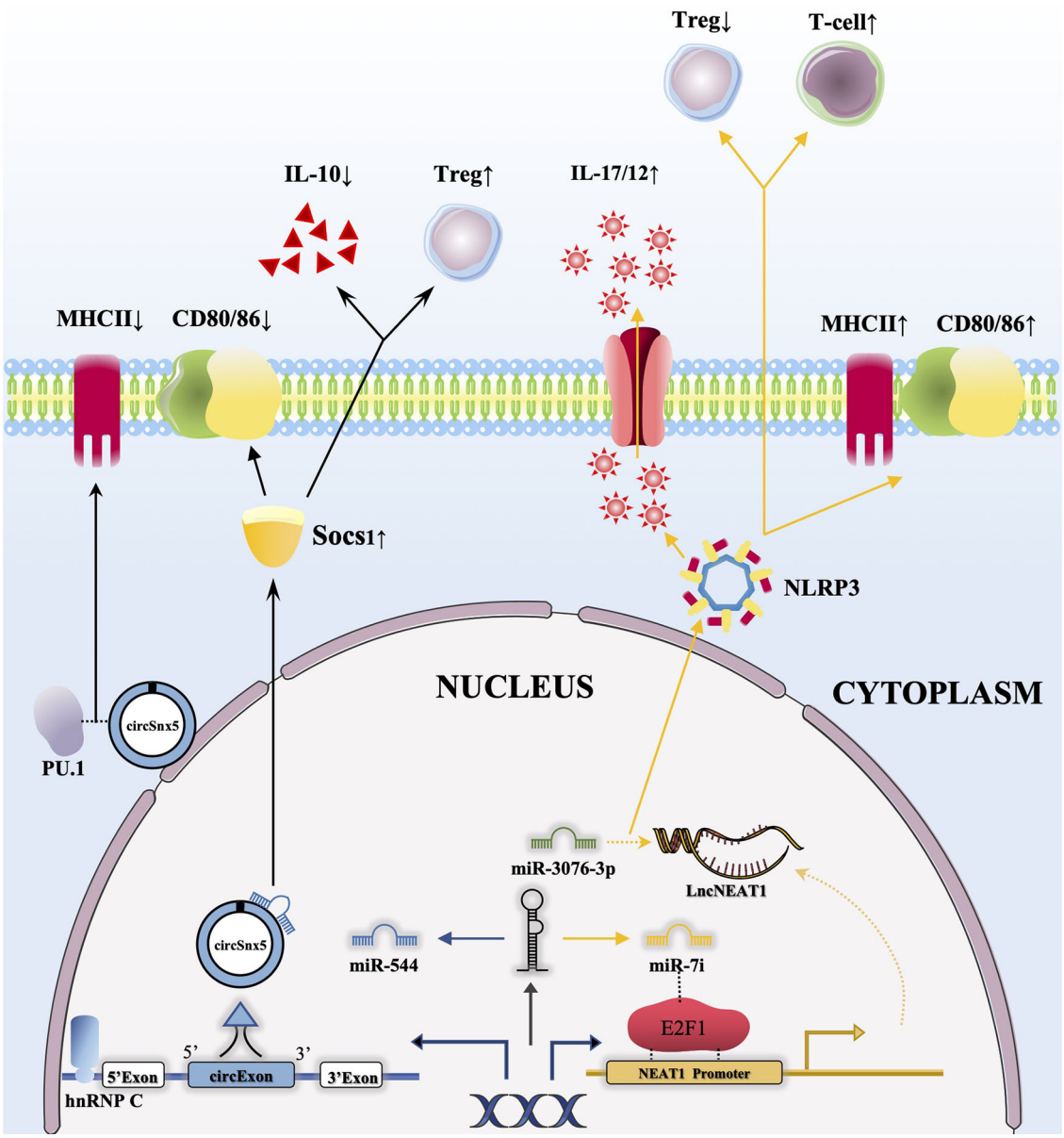


FIGURE 2 | CircRNAs and lncRNAs regulate the function of DCs through the ceRNA network. 1) The combination of hnRNP C with circSnx5 promotes the expression of circSnx5 in DCs, and circSnx5 sponging with miR-544 reduces the inhibitory effect of miR-544 on Socs1, thus reducing the expression of CD80/86 and the secretion of IL-10 and increasing the number of Tregs. In addition, circSnx5 combined with PU.1 can directly reduce the expression of MHCII; 2) miRNA let-7i can regulate the expression of lncNEAT1 by binding E2F1, and lncNEAT1 is able to regulate NLRP3 inflammasome by inhibiting Mir-3076-3P, then increasing expression of MHCII/CD80/86, promoting secretion of IL-17/12 as well as reducing the number of Tregs and increasing the activation of T cells.

enhance the effect of glucocorticoids on SLE by promoting pDC apoptosis (25). In addition, miR-142-3p, miR-363 and miR-29a change the proliferation level of Treg and T cells through regulation of DCs and then affect the level of inflammation in related autoimmune diseases (29, 39, 77). For the other two types of non-coding RNA (circRNA, lncRNA), representatively, CircSnx5 and lncNEAT1 can bind miRNA *via* a ceRNA network and change the inflammatory phenotypes of DCs in related autoimmune diseases (41, 44). In general, knockdown or overexpression of non-coding RNAs may be a novel potential therapeutic strategy for related autoimmune and tolerance-related diseases. In the development process of different autoimmune and tolerance-related diseases, it is of great potential to further understand the abnormal expression of non-coding RNAs and the regulation of these diseases through DCs, which can bring new therapeutic targets or strategies for these complex ones.

CONCLUSION AND FUTURE PERSPECTIVES

Dendritic cells (DCs), typical APCs in the human body, play an important role in connecting innate immunity and adaptive immunity and affect the pathological mechanism of various immune diseases. Our understanding of non-coding RNAs has changed, and now, instead of being considered "junk" transcription products, they are recognized as functional regulators that mediate various cellular processes. This review highlights the regulatory effects and potential therapeutic targets targeted by DCs of abnormally expressed non-coding RNAs (miRNAs, lncRNAs, circRNAs) in autoimmune diseases and immune tolerance diseases. Although non-coding RNAs have been proven to be potential diagnostic and prognostic biomarkers, the specificity and sensitivity of most existing noncoding RNA biomarkers are still insufficient for clinical application. Further large-scale prospective clinical trials will validate and promote the clinical application of noncoding RNA biomarker candidates. Furthermore, the number and profundity of studies on the effects of lncRNAs and circRNAs on DCs in these diseases remain scarce. Despite these defects, further research on the regulatory mechanisms of non-coding RNA in target cells in specific diseases may provide a more solid foundation for diagnostic and therapeutic research in autoimmune diseases and immune tolerance diseases.

AUTHOR CONTRIBUTIONS

YL wrote the original draft, table and figure preparation. XW contributed to the conception and design of the study. FY and YZ: literature query. TY: editing. LY: supervision, review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (No. 81770568 to LY).

REFERENCES

1. Steinman RM, Kaplan G, Witmer MD, Cohn ZA. Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice. V. Purification of Spleen Dendritic Cells, New Surface Markers, and Maintenance *In Vitro*. *J Exp Med* (1979) 149(1):1–16. doi: 10.1084/jem.149.1.1
2. Unanue ER, Cerottini JC. The Immunogenicity of Antigen Bound to the Plasma Membrane of Macrophages. *J Exp Med* (1970) 131(4):711–25. doi: 10.1084/jem.131.4.711
3. Puhr S, Lee J, Zvezdova E, Zhou YJ, Liu K. Dendritic Cell Development-History, Advances, and Open Questions. *Semin Immunol* (2015) 27(6):388–96. doi: 10.1016/j.smim.2016.03.012
4. Allman D, Dalod M, Asselin-Paturel C, Delale T, Robbins SH, Trinchieri G, et al. Ikaros Is Required for Plasmacytoid Dendritic Cell Differentiation. *Blood* (2006) 108(13):4025–34. doi: 10.1182/blood-2006-03-007757
5. Carotta S, Dakic A, D'Amico A, Pang SH, Greig KT, Nutt SL, et al. The Transcription Factor PU.1 Controls Dendritic Cell Development and Flt3 Cytokine Receptor Expression in a Dose-Dependent Manner. *Immunity* (2010) 32(5):628–41. doi: 10.1016/j.immuni.2010.05.005
6. Onodera K, Fujiwara T, Onishi Y, Itoh-Nakadai A, Okitsu Y, Fukuhara N, et al. GATA2 Regulates Dendritic Cell Differentiation. *Blood* (2016) 128 (4):508–18. doi: 10.1182/blood-2016-02-698118
7. Rathinam C, Geffers R, Yücel R, Buer J, Welte K, Möröy T, et al. The Transcriptional Repressor Gfi1 Controls STAT3-Dependent Dendritic Cell Development and Function. *Immunity* (2005) 22(6):717–28. doi: 10.1016/j.immuni.2005.04.007
8. Collin M, Bigley V. Human Dendritic Cell Subsets: An Update. *Immunology* (2018) 154(1):3–20. doi: 10.1111/imm.12888
9. Brown CC, Gudjonson H, Pritykin Y, Deep D, Lavallée VP, Mendoza A, et al. Transcriptional Basis of Mouse and Human Dendritic Cell Heterogeneity. *Cell* (2019) 179(4):846–63. doi: 10.1016/j.cell.2019.09.035
10. Bonasio R, Scimone ML, Schaerli P, Grabie N, Lichtman AH, von Andrian UH. Clonal Deletion of Thymocytes by Circulating Dendritic Cells Homing to the Thymus. *Nat Immunol* (2006) 7(10):1092–100. doi: 10.1038/ni1385
11. Ohnmacht C, Pullner A, King SB, Drexler I, Meier S, Brocker T, et al. Constitutive Ablation of Dendritic Cells Breaks Self-Tolerance of CD4 T Cells and Results in Spontaneous Fatal Autoimmunity. *J Exp Med* (2009) 206 (3):549–59. doi: 10.1084/jem.20082394
12. Mahnke K, Qian Y, Knop J, Enk AH. Induction of CD4+/CD25+ Regulatory T Cells by Targeting of Antigens to Immature Dendritic Cells. *Blood* (2003) 101(12):4862–9. doi: 10.1182/blood-2002-10-3229
13. Bejerano G, Pheasant M, Makunin I, Stephen S, Kent WJ, Mattick JS, et al. Ultraconserved Elements in the Human Genome. *Science* (2004) 304 (5675):1321–5. doi: 10.1126/science.1098119
14. Kim SS, Lee SV. Non-Coding RNAs in Caenorhabditis Elegans Aging. *Mol Cells* (2019) 42(5):379–85. doi: 10.14348/molcells.2019.0077
15. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin Signature Reveals Over a Thousand Highly Conserved Large Non-Coding RNAs in Mammals. *Nature* (2009) 458(7235):223–7. doi: 10.1038/nature07672
16. Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, et al. The GENCODE V7 Catalog of Human Long Noncoding RNAs: Analysis of Their Gene Structure, Evolution, and Expression. *Genome Res* (2012) 22 (9):1775–89. doi: 10.1101/gr.132159.111
17. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, et al. Landscape of Transcription in Human Cells. *Nature* (2012) 489(7414):101–8. doi: 10.1038/nature11233
18. Feingold EA, Good PJ, Guyer MS, Kamholz S, Liefer L, Wetterstrand K, et al. The ENCODE (Encyclopedia Of Dna Elements) Project. *Science* (2004) 306 (5696):636–40. doi: 10.1126/science.1105136
19. Kuipers H, Schnorfeil FM, Fehling HJ, Bartels H, Brocker T. Dicer-Dependent microRNAs Control Maturation, Function, and Maintenance of Langerhans Cells *In Vivo*. *J Immunol* (2010) 185(1):400–9. doi: 10.4049/jimmunol.0903912
20. Wang P, Xue Y, Han Y, Lin L, Wu C, Xu S, et al. The STAT3-binding Long Noncoding RNA lnc-DC Controls Human Dendritic Cell Differentiation. *Science* (2014) 344(6181):310–3. doi: 10.1126/science.1251456
21. Zhang Y, Zhang G, Liu Y, Chen R, Zhao D, McAlister V, et al. Gdf15 Regulates Malat-1 Circular RNA and Inactivates Nfκb Signaling Leading to Immune Tolerogenic DCs for Preventing Alloimmune Rejection in Heart Transplantation. *Front Immunol* (2018) 9:2407. doi: 10.3389/fimmu.2018.02407
22. Salvi V, Gianello V, Busatto S, Bergese P, Andreoli L, D'Oro U, et al. Exosome-Delivered microRNAs Promote IFN-α Secretion by Human Plasmacytoid DCs Via TLR7. *JCI Insight* (2018) 3(10). doi: 10.1172/jci.insight.98204
23. van den Hoogen LL, Rossato M, Lopes AP, Pandit A, Bekker CPJ, Fritsch-Stork RDE, et al. microRNA Downregulation in Plasmacytoid Dendritic Cells in Interferon-Positive Systemic Lupus Erythematosus and Antiphospholipid Syndrome. *Rheumatol (Oxford)* (2018) 57(9):1669–74. doi: 10.1093/rheumatology/key159

24. Yan S, Yim LY, Tam RC, Chan A, Lu L, Lau CS, et al. Microrna-155 Mediates Augmented CD40 Expression in Bone Marrow Derived Plasmacytoid Dendritic Cells in Symptomatic Lupus-Prone NZB/W F1 Mice. *Int J Mol Sci* (2016) 17(8):1282. doi: 10.3390/ijms17081282
25. Hong Y, Wu J, Zhao J, Wang H, Liu Y, Chen T, et al. miR-29b and miR-29c Are Involved in Toll-Like Receptor Control of Glucocorticoid-Induced Apoptosis in Human Plasmacytoid Dendritic Cells. *PloS One* (2013) 8(7): e69926. doi: 10.1371/journal.pone.0069926
26. Gao S, Yuan L, Wang Y, Hua C. Enhanced Expression of TREM-1 in Splenic cDCs in Lupus Prone Mice and it Was Modulated by miRNA-150. *Mol Immunol* (2017) 81:127–34. doi: 10.1016/j.molimm.2016.12.006
27. Wang Y, Liang J, Qin H, Ge Y, Du J, Lin J, et al. Elevated Expression of miR-142-3p Is Related to the Pro-Inflammatory Function of Monocyte-Derived Dendritic Cells in SLE. *Arthritis Res Ther* (2016) 18(1):263. doi: 10.1186/s13075-016-1158-z
28. Kurowska-Stolarska M, Alivernini S, Melchor EG, Elmesmari A, Tolusso B, Tange C, et al. MicroRNA-34a Dependent Regulation of AXL Controls the Activation of Dendritic Cells in Inflammatory Arthritis. *Nat Commun* (2017) 8:15877. doi: 10.1038/ncomms15877
29. Pan F, Xiang H, Yan J, Hong L, Zhang L, Liu Y, et al. Dendritic Cells From Rheumatoid Arthritis Patient Peripheral Blood Induce Th17 Cell Differentiation Via miR-363/Integrin αv/TGF-β Axis. *Scand J Immunol* (2017) 85(6):441–9. doi: 10.1111/sji.12550
30. Hillen MR, Chouri E, Wang M, Blokland SLM, Hartgring SAY, Concepcion AN, et al. Dysregulated miRNome of Plasmacytoid Dendritic Cells From Patients With Sjögren's Syndrome Is Associated With Processes at the Centre of Their Function. *Rheumatol (Oxf)* (2019) 58(12):2305–14. doi: 10.1093/rheumatology/kez195
31. Lopes AP, van Roon JAG, Blokland SLM, Wang M, Chouri E, Hartgring SAY, et al. MicroRNA-130a Contributes to Type-2 Classical DC-activation in Sjögren's Syndrome by Targeting Mitogen- and Stress-Activated Protein Kinase-1. *Front Immunol* (2019) 10:1335. doi: 10.3389/fimmu.2019.01335
32. Wu W, He C, Liu C, Cao AT, Xue X, Evans-Marin HL, et al. miR-10a Inhibits Dendritic Cell Activation and Th1/Th17 Cell Immune Responses in IBD. *Gut* (2015) 64(11):1755–64. doi: 10.1136/gutjnl-2014-307980
33. Ifergan I, Chen S, Zhang B, Miller SD. Cutting Edge: Microrna-223 Regulates Myeloid Dendritic Cell-Driven Th17 Responses in Experimental Autoimmune Encephalomyelitis. *J Immunol* (2016) 196(4):1455–9. doi: 10.4049/jimmunol.1501965
34. Hoye ML, Archambault AS, Gordon TM, Oetjen LK, Cain MD, Klein RS, et al. MicroRNA Signature of Central Nervous System-Infiltrating Dendritic Cells in an Animal Model of Multiple Sclerosis. *Immunology* (2018) 155 (1):112–22. doi: 10.1111/imm.12934
35. Rossato M, Affandi AJ, Thordardottir S, Wichers CGK, Cossu M, Broen JCA, et al. Association of MicroRNA-618 Expression With Altered Frequency and Activation of Plasmacytoid Dendritic Cells in Patients With Systemic Sclerosis. *Arthritis Rheumatol* (2017) 69(9):1891–902. doi: 10.1002/art.40163
36. Chen L, Hou X, Zhang M, Zheng Y, Zheng X, Yang Q, et al. MicroRNA-223-3p Modulates Dendritic Cell Function and Ameliorates Experimental Autoimmune Myocarditis by Targeting the NLRP3 Inflammasome. *Mol Immunol* (2020) 117:73–83. doi: 10.1016/j.molimm.2019.10.027
37. Chen S, Smith BA, Iype J, Prestipino A, Pfeifer D, Grundmann S, et al. MicroRNA-155-Deficient Dendritic Cells Cause Less Severe GVHD Through Reduced Migration and Defective Inflammasome Activation. *Blood* (2015) 126(1):103–12. doi: 10.1182/blood-2014-12-617258
38. Stickel N, Hanke K, Marschner D, Prinz G, Köhler M, Melchinger W, et al. MicroRNA-146a Reduces MHC-II Expression Via Targeting JAK/STAT Signaling in Dendritic Cells After Stem Cell Transplantation. *Leukemia* (2017) 31(12):2732–41. doi: 10.1038/leu.2017.137
39. Ranganathan P, Ngankeu A, Zitzer NC, Leoncini P, Yu X, Casadei L, et al. Serum Mir-29a Is Upregulated in Acute Graft-*Versus*-Host Disease and Activates Dendritic Cells Through TLR Binding. *J Immunol* (2017) 198 (6):2500–12. doi: 10.4049/jimmunol.1601778
40. Wang Y, Chen S, Chen S, Du J, Lin J, Qin H, et al. Long Noncoding RNA Expression Profile and Association With SLEDAI Score in Monocyte-Derived Dendritic Cells From Patients With Systematic Lupus Erythematosus. *Arthritis Res Ther* (2018) 20(1):138. doi: 10.1186/s13075-018-1640-x
41. Zhang M, Zheng Y, Sun Y, Li S, Chen L, Jin X, et al. Knockdown of NEAT1 Induces Tolerogenic Phenotype in Dendritic Cells by Inhibiting Activation of NLRP3 Inflammasome. *Theranostics* (2019) 9(12):3425–42. doi: 10.7150/thno.33178
42. Wu J, Zhang H, Zheng Y, Jin X, Liu M, Li S, et al. The Long Noncoding RNA Malat1 Induces Tolerogenic Dendritic Cells and Regulatory T Cells Via Mir155/Dendritic Cell-Specific Intercellular Adhesion Molecule-3 Grabbing Nonintegrin/Il10 Axis. *Front Immunol* (2018) 9:1847. doi: 10.3389/fimmu.2018.01847
43. Luan J, Jiao C, Kong W, Fu J, Qu W, Chen Y, et al. Circhla-C Plays an Important Role in Lupus Nephritis by Sponging Mir-150. *Mol Ther Nucleic Acids* (2018) 10:245–53. doi: 10.1016/j.omtn.2017.12.006
44. Chen Q, Mang G, Wu J, Sun P, Li T, Zhang H, et al. Circular RNA Circsnx5 Controls Immunogenicity of Dendritic Cells Through the Mir-544/SOCS1 Axis and PU.1 Activity Regulation. *Mol Ther* (2020) 28(11):2503–18. doi: 10.1016/j.ymthe.2020.07.001
45. Merad M, Sathe P, Helft J, Miller J, Mortha A. The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. *Annu Rev Immunol* (2013) 31:563–604. doi: 10.1146/annurev-immunol-020711-074950
46. Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid Dendritic Cells (Natural Interferon- Alpha/Beta-Producing Cells) Accumulate in Cutaneous Lupus Erythematosus Lesions. *Am J Pathol* (2001) 159(1):237–43. doi: 10.1016/s0002-9440(10)61689-6
47. Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, et al. Plasmacytoid Predendritic Cells Initiate Psoriasis Through Interferon-Alpha Production. *J Exp Med* (2005) 202(1):135–43. doi: 10.1084/jem.20050500
48. Sozzani S, Vermi W, Del Prete A, Facchetti F. Trafficking Properties of Plasmacytoid Dendritic Cells in Health and Disease. *Trends Immunol* (2010) 31(7):270–7. doi: 10.1016/j.it.2010.05.004
49. Soni C, Perez OA, Voss WN, Pucella JN, Serpas L, Mehl J, et al. Plasmacytoid Dendritic Cells and Type I Interferon Promote Extrafollicular B Cell Responses to Extracellular Self-Dna. *Immunity* (2020) 52(6):1022–38.e7. doi: 10.1016/j.immuni.2020.04.015
50. Barrat FJ, Su L. A Pathogenic Role of Plasmacytoid Dendritic Cells in Autoimmunity and Chronic Viral Infection. *J Exp Med* (2019) 216(9):1974–85. doi: 10.1084/jem.20181359
51. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic Cells, Monocytes and Macrophages: A Unified Nomenclature Based on Ontogeny. *Nat Rev Immunol* (2014) 14(8):571–8. doi: 10.1038/nri3712
52. Sichien D, Lambrecht BN, Guilliams M, Scott CL. Development of Conventional Dendritic Cells: From Common Bone Marrow Progenitors to Multiple Subsets in Peripheral Tissues. *Mucosal Immunol* (2017) 10 (4):831–44. doi: 10.1038/mi.2017.8
53. Worbs T, Hammerschmidt SI, Förster R. Dendritic Cell Migration in Health and Disease. *Nat Rev Immunol* (2017) 17(1):30–48. doi: 10.1038/nri.2016.116
54. Gill MA, Blanco P, Arce E, Pascual V, Banchereau J, Palucka AK. Blood Dendritic Cells and DC-poietins in Systemic Lupus Erythematosus. *Hum Immunol* (2002) 63(12):1172–80. doi: 10.1016/s0198-8859(02)00756-5
55. Migita K, Miyashita T, Maeda Y, Kimura H, Nakamura M, Yatsuhashi H, et al. Reduced Blood BDCA-2+ (Lymphoid) and CD11c+ (Myeloid) Dendritic Cells in Systemic Lupus Erythematosus. *Clin Exp Immunol* (2005) 142(1):84–91. doi: 10.1111/j.1365-2249.2005.02897.x
56. Jin O, Kavikondala S, Sun L, Fu R, Mok MY, Chan A, et al. Systemic Lupus Erythematosus Patients Have Increased Number of Circulating Plasmacytoid Dendritic Cells, But Decreased Myeloid Dendritic Cells With Deficient CD83 Expression. *Lupus* (2008) 17(7):654–62. doi: 10.1177/0961203308089410
57. Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP, et al. Enumeration and Phenotypical Analysis of Distinct Dendritic Cell Subsets in Psoriatic Arthritis and Rheumatoid Arthritis. *Arthritis Res Ther* (2006) 8(1): R15. doi: 10.1186/ar1864
58. Page G, Miossec P. Paired Synovium and Lymph Nodes From Rheumatoid Arthritis Patients Differ in Dendritic Cell and Chemokine Expression. *J Pathol* (2004) 204(1):28–38. doi: 10.1002/path.1607
59. Power CA, Church DJ, Meyer A, Alouani S, Proudfoot AE, Clark-Lewis I, et al. Cloning and Characterization of a Specific Receptor for the Novel CC

Chemokine MIP-3alpha From Lung Dendritic Cells. *J Exp Med* (1997) 186 (6):825–35. doi: 10.1084/jem.186.6.825
60. Fan X, Men R, Huang C, Shen M, Wang T, Ghnewa Y, et al. Critical Roles of Conventional Dendritic Cells in Autoimmune Hepatitis Via Autophagy Regulation. *Cell Death Dis* (2020) 11(1):23. doi: 10.1038/s41419-019-2217-6
61. Segura E, Amigorena S. Inflammatory Dendritic Cells in Mice and Humans. *Trends Immunol* (2013) 34(9):440–5. doi: 10.1016/j.it.2013.06.001
62. Takenaka MC, Quintana FJ. Tolerogenic Dendritic Cells. *Semin Immunopathol* (2017) 39(2):113–20. doi: 10.1007/s00281-016-0587-8
63. Audiger C, Rahman MJ, Yun TJ, Tarbell KV, Lesage S. The Importance of Dendritic Cells in Maintaining Immune Tolerance. *J Immunol* (2017) 198 (6):2223–31. doi: 10.4049/jimmunol.1601629
64. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T Cells: Mechanisms of Differentiation and Function. *Annu Rev Immunol* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
65. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary Dendritic Cells Producing IL-10 Mediate Tolerance Induced by Respiratory Exposure to Antigen. *Nat Immunol* (2001) 2(8):725–31. doi: 10.1038/90667
66. Mascanfroni ID, Yeste A, Vieira SM, Burns EJ, Patel B, Sloma I, et al. Il-27 Acts on DCs to Suppress the T Cell Response and Autoimmunity by Inducing Expression of the Immunoregulatory Molecule CD39. *Nat Immunol* (2013) 14(10):1054–63. doi: 10.1038/ni.2695
67. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, et al. Potential Regulatory Function of Human Dendritic Cells Expressing Indoleamine 2,3-Dioxygenase. *Science* (2002) 297(5588):1867–70. doi: 10.1126/science.1073514
68. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, et al. Loss of Integrin Alpha(V)Beta8 on Dendritic Cells Causes Autoimmunity and Colitis in Mice. *Nature* (2007) 449(7160):361–5. doi: 10.1038/nature06110
69. Morante-Palacios O, Fondelli F, Ballestar E, Martínez-Cáceres EM. Tolerogenic Dendritic Cells in Autoimmunity and Inflammatory Diseases. *Trends Immunol* (2021) 42(1):59–75. doi: 10.1016/j.it.2020.11.001
70. Marin E, Bouchet-Delbos L, Renoult O, Louvet C, Nerriere-Daguin V, Managh AJ, et al. Human Tolerogenic Dendritic Cells Regulate Immune Responses Through Lactate Synthesis. *Cell Metab* (2019) 30(6):1075–90.e8. doi: 10.1016/j.cmet.2019.11.011
71. Kim VN, Han J, Siomi MC. Biogenesis of Small RNAs in Animals. *Nat Rev Mol Cell Biol* (2009) 10(2):126–39. doi: 10.1038/nrm2632
72. Niu L, Lou F, Sun Y, Sun L, Cai X, Liu Z, et al. A Micropeptide Encoded by Lncrna MIR155HG Suppresses Autoimmune Inflammation Via Modulating Antigen Presentation. *Sci Adv* (2020) 6(21):eaaz2059. doi: 10.1126/sciadv.aaz2059
73. Tsokos GC, Lo MS, Costa Reis P, Sullivan KE. New Insights Into the Immunopathogenesis of Systemic Lupus Erythematosus. *Nat Rev Rheumatol* (2016) 12(12):716–30. doi: 10.1038/nrrheum.2016.186
74. Furie R, Werth VP, Merola JF, Stevenson L, Reynolds TL, Naik H, et al. Monoclonal Antibody Targeting BDCA2 Ameliorates Skin Lesions in Systemic Lupus Erythematosus. *J Clin Invest* (2019) 129(3):1359–71. doi: 10.1172/jci124466
75. Li J, Gong J, Li P, Li M, Liu Y, Liang S, et al. Knockdown of microRNA-155 in Kupffer Cells Results in Immunosuppressive Effects and Prolongs Survival of Mouse Liver Allografts. *Transplantation* (2014) 97(6):626–35. doi: 10.1097/tp.0000000000000061
76. Chyuan IT, Tzeng HT, Chen JY. Signaling Pathways of Type I and Type Iii Interferons and Targeted Therapies in Systemic Lupus Erythematosus. *Cells* (2019) 8(9):963. doi: 10.3390/cells8090963
77. Molad Y, Pokroy-Shapira E, Kaptzan T, Monselise A, Shalita-Chesner M, Monselise Y. Serum Soluble Triggering Receptor on Myeloid Cells-1 (sTREM-1) Is Elevated in Systemic Lupus Erythematosus But Does Not Distinguish Between Lupus Alone and Concurrent Infection. *Inflammation* (2013) 36(6):1519–24. doi: 10.1007/s10753-013-9694-z
78. Funes SC, Ríos M, Gómez-Santander F, Fernández-Fierro A, Altamirano-Lagos MJ, Rivera-Perez D, et al. Tolerogenic Dendritic Cell Transfer Ameliorates Systemic Lupus Erythematosus in Mice. *Immunology* (2019) 158(4):322–39. doi: 10.1111/imm.13119
79. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The Global Burden of Rheumatoid Arthritis: Estimates From the Global Burden of Disease 2010 Study. *Ann Rheum Dis* (2014) 73(7):1316–22. doi: 10.1136/annrheumdis-2013-204627
80. Crowson CS, Matteson EL, Myasoedova E, Michet CJ, Ernste FC, Warrington KJ, et al. The Lifetime Risk of Adult-Onset Rheumatoid Arthritis and Other Inflammatory Autoimmune Rheumatic Diseases. *Arthritis Rheum* (2011) 63(3):633–9. doi: 10.1002/art.30155
81. Moret FM, Hack CE, van der Wurff-Jacobs KM, de Jager W, Radstake TR, Lafeber FP, et al. Intra-Articular CD1c-Expressing Myeloid Dendritic Cells From Rheumatoid Arthritis Patients Express a Unique Set of T Cell-Attracting Chemokines and Spontaneously Induce Th1, Th17 and Th2 Cell Activity. *Arthritis Res Ther* (2013) 15(5):R155. doi: 10.1186/ar4338
82. Sacre SM, Lo A, Gregory B, Simmonds RE, Williams L, Feldmann M, et al. Inhibitors of TLR8 Reduce TNF Production From Human Rheumatoid Synovial Membrane Cultures. *J Immunol* (2008) 181(11):8002–9. doi: 10.4049/jimmunol.181.11.8002
83. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. Development, Cytokine Profile and Function of Human Interleukin 17-Producing Helper T Cells. *Nat Immunol* (2007) 8(9):950–7. doi: 10.1038/ni1497
84. Malladi AS, Sack KE, Shiboski SC, Shiboski CH, Baer AN, Banushree R, et al. Primary Sjögren's Syndrome as a Systemic Disease: A Study of Participants Enrolled in an International Sjögren's Syndrome Registry. *Arthritis Care Res (Hoboken)* (2012) 64(6):911–8. doi: 10.1002/acr.21610
85. Vogelsang P, Brun JG, Oijordsbakken G, Skarstein K, Jonsson R, Appel S. Levels of Plasmacytoid Dendritic Cells and Type-2 Myeloid Dendritic Cells Are Reduced in Peripheral Blood of Patients With Primary Sjogren's Syndrome. *Ann Rheum Dis* (2010) 69(6):1235–8. doi: 10.1136/ard.2009.118158
86. Gottenberg JE, Cagnard N, Lucchesi C, Letourneur F, Mistou S, Lazure T, et al. Activation of IFN Pathways and Plasmacytoid Dendritic Cell Recruitment in Target Organs of Primary Sjögren's Syndrome. *Proc Natl Acad Sci USA* (2006) 103(8):2770–5. doi: 10.1073/pnas.0510837103
87. Zhao J, Kubo S, Nakayamada S, Shimajiri S, Zhang X, Yamaoka K, et al. Association of Plasmacytoid Dendritic Cells With B Cell Infiltration in Minor Salivary Glands in Patients With Sjögren's Syndrome. *Mod Rheumatol* (2016) 26(5):716–24. doi: 10.3109/14397595.2015.1129694
88. Boulé MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Rifkin IR. Toll-Like Receptor 9-Dependent and -Independent Dendritic Cell Activation by Chromatin-Immunoglobulin G Complexes. *J Exp Med* (2004) 199(12):1631–40. doi: 10.1084/jem.20031942
89. Wang-Renault SF, Boudaoud S, Nocturne G, Roche E, Sigrist N, Daviaud C, et al. Deregulation of microRNA Expression in Purified T and B Lymphocytes From Patients With Primary Sjögren's Syndrome. *Ann Rheum Dis* (2018) 77(1):133–40. doi: 10.1136/annrheumdis-2017-211417
90. Alevizos I, Alexander S, Turner RJ, Illei GG. MicroRNA Expression Profiles as Biomarkers of Minor Salivary Gland Inflammation and Dysfunction in Sjögren's Syndrome. *Arthritis Rheum* (2011) 63(2):535–44. doi: 10.1002/art.30131
91. Brito-Zerón P, Baldini C, Bootsma H, Bowman SJ, Jonsson R, Mariette X, et al. Sjögren Syndrome. *Nat Rev Dis Primers* (2016) 2:16047. doi: 10.1038/nrdp.2016.47
92. Kiripolsky J, McCabe LG, Kramer JM. Innate Immunity in Sjögren's Syndrome. *Clin Immunol* (2017) 182:4–13. doi: 10.1016/j.clim.2017.04.003
93. Peng H, Guerau-de-Arellano M, Mehta VB, Yang Y, Huss DJ, Papenfuss TL, et al. Dimethyl Fumarate Inhibits Dendritic Cell Maturation Via Nuclear Factor κb (Nf-κb) and Extracellular Signal-Regulated Kinase 1 and 2 (ERK1/2) and Mitogen Stress-Activated Kinase 1 (MSK1) Signaling. *J Biol Chem* (2012) 287(33):28017–26. doi: 10.1074/jbc.M112.383380
94. Mackie RI, Sghir A, Gaskins HR. Developmental Microbial Ecology of the Neonatal Gastrointestinal Tract. *Am J Clin Nutr* (1999) 69(5):1035s–45s. doi: 10.1093/ajcn/69.5.1035s
95. Taganov KD, Boldin MP, Chang KJ, Baltimore D. Nf-kappaB-dependent Induction of microRNA miR-146, an Inhibitor Targeted to Signaling Proteins of Innate Immune Responses. *Proc Natl Acad Sci USA* (2006) 103 (33):12481–6. doi: 10.1073/pnas.0605298103
96. Niess JH, Reinecker HC. Lamina Propria Dendritic Cells in the Physiology and Pathology of the Gastrointestinal Tract. *Curr Opin Gastroenterol* (2005) 21(6):687–91. doi: 10.1097/01.mog.0000181710.96904.58
97. Xue X, Feng T, Yao S, Wolf KJ, Liu CG, Liu X, et al. Microbiota Downregulates Dendritic Cell Expression of miR-10a, Which Targets IL-

12/IL-23p40. *J Immunol* (2011) 187(11):5879–86. doi: 10.4049/jimmunol.1100535
98. Burton PR, Clayton DG, Cardon LR, Craddock N, Deloukas P, Duncanson A, et al. Genome-Wide Association Study of 14,000 Cases of Seven Common Diseases and 3,000 Shared Controls. *Nature* (2007) 447(7145):661–78. doi: 10.1038/nature05911
99. Szperl A, Saavalainen P, Weersma RK, Lappalainen M, Paavola-Sakki P, Halme L, et al. Functional Polymorphism in IL12B Promoter Site Is Associated With Ulcerative Colitis. *Inflammation Bowel Dis* (2011) 17(6):E38–40. doi: 10.1002/ibd.21670
100. Kanneganti TD, Lamkanfi M, Núñez G. Intracellular NOD-Like Receptors in Host Defense and Disease. *Immunity* (2007) 27(4):549–59. doi: 10.1016/j.immuni.2007.10.002
101. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A Frameshift Mutation in NOD2 Associated With Susceptibility to Crohn's Disease. *Nature* (2001) 411(6837):603–6. doi: 10.1038/35079114
102. Bailey SL, Schreiner B, McMahon EJ, Miller SD. CNS Myeloid DCs Presenting Endogenous Myelin Peptides 'Preferentially' Polarize CD4+ T (H)-17 Cells in Relapsing EAE. *Nat Immunol* (2007) 8(2):172–80. doi: 10.1038/ni1430
103. King IL, Dickendesher TL, Segal BM. Circulating Ly-6C+ Myeloid Precursors Migrate to the CNS and Play a Pathogenic Role During Autoimmune Demyelinating Disease. *Blood* (2009) 113(14):3190–7. doi: 10.1182/blood-2008-07-168575
104. Junker A, Krumbholz M, Eisele S, Mohan H, Augstein F, Bittner R, et al. MicroRNA Profiling of Multiple Sclerosis Lesions Identifies Modulators of the Regulatory Protein CD47. *Brain* (2009) 132(Pt 12):3342–52. doi: 10.1093/brain/awp300
105. Ah Kioon MD, Tripodo C, Fernandez D, Kirou KA, Spiera RF, Crow MK, et al. Plasmacytoid Dendritic Cells Promote Systemic Sclerosis With a Key Role for TLR8. *Sci Transl Med* (2018) 10(423). doi: 10.1126/scitranslmed.aam8458
106. Jaen O, Rullé S, Bessis N, Zago A, Boissier MC, Falgarone G. Dendritic Cells Modulated by Innate Immunity Improve Collagen-Induced Arthritis and Induce Regulatory T Cells *In Vivo*. *Immunology* (2009) 126(1):35–44. doi: 10.1111/j.1365-2567.2008.02875.x
107. Ke Y, Liu K, Huang GQ, Cui Y, Kaplan HJ, Shao H, et al. Anti-Inflammatory Role of IL-17 in Experimental Autoimmune Uveitis. *J Immunol* (2009) 182(5):3183–90. doi: 10.4049/jimmunol.0802487
108. Quan MY, Song XJ, Liu HJ, Deng XH, Hou HQ, Chen LP, et al. Amlexanox Attenuates Experimental Autoimmune Encephalomyelitis by Inhibiting Dendritic Cell Maturation and Reprogramming Effector and Regulatory T Cell Responses. *J Neuroinflamm* (2019) 16(1):52. doi: 10.1186/s12974-019-1438-z
109. Zeiser R, Blazar BR. Acute Graft-*versus*-Host Disease - Biologic Process, Prevention, and Therapy. *N Engl J Med* (2017) 377(22):2167–79. doi: 10.1056/NEJMra1609337
110. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray Analysis Shows That Some MicroRNAs Downregulate Large Numbers of Target mRNAs. *Nature* (2005) 433(7027):769–73. doi: 10.1038/nature03315
111. Rinn JL, Chang HY. Genome Regulation by Long Noncoding RNAs. *Annu Rev Biochem* (2012) 81:145–66. doi: 10.1146/annurev-biochem-051410-092902
112. Sigova AA, Mullen AC, Molinie B, Gupta S, Orlando DA, Guenther MG, et al. Divergent Transcription of Long Noncoding RNA/mRNA Gene Pairs in Embryonic Stem Cells. *Proc Natl Acad Sci USA* (2013) 110(8):2876–81. doi: 10.1073/pnas.1221904110
113. Spurlock CF3rd, Tossberg JT, Guo Y, Collier SP, Crooke PS3rd, Aune TM. Expression and Functions of Long Noncoding RNAs During Human T Helper Cell Differentiation. *Nat Commun* (2015) 6:6932. doi: 10.1038/ncomms7932
114. Chen YG, Satpathy AT, Chang HY. Gene Regulation in the Immune System by Long Noncoding Rnas. *Nat Immunol* (2017) 18(9):962–72. doi: 10.1038/ni.3771
115. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO. Circular RNAs are the Predominant Transcript Isoform From Hundreds of Human Genes in Diverse Cell Types. *PloS One* (2012) 7(2):e30733. doi: 10.1371/journal.pone.0030733
116. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, et al. Circular RNAs Are Abundant, Conserved, and Associated With ALU Repeats. *Rna* (2013) 19(2):141–57. doi: 10.1261/rna.035667.112
117. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, et al. Circular RNAs Are a Large Class of Animal RNAs With Regulatory Potency. *Nature* (2013) 495(7441):333–8. doi: 10.1038/nature11928
118. Li H, Li K, Lai W, Li X, Wang H, Yang J, et al. Comprehensive Circular RNA Profiles in Plasma Reveals That Circular RNAs Can be Used as Novel Biomarkers for Systemic Lupus Erythematosus. *Clin Chim Acta* (2018) 480:17–25. doi: 10.1016/j.cca.2018.01.026
119. Li LJ, Huang Q, Pan HF, Ye DQ. Circular RNAs and Systemic Lupus Erythematosus. *Exp Cell Res* (2016) 346(2):248–54. doi: 10.1016/j.yexcr.2016.07.021
120. Zhang C, Wang X, Chen Y, Wu Z, Zhang C, Shi W. The Down-Regulation of Hsa_Circ_0012919, the Sponge for miR-125a-3p, Contributes to DNA Methylation of CD11a and CD70 in CD4(+) T Cells of Systemic Lupus Erythematous. *Clin Sci (Lond)* (2018) 132(21):2285–98. doi: 10.1042/cs20180403
121. Xu Z, Li P, Fan L, Wu M. The Potential Role of circRNA in Tumor Immunity Regulation and Immunotherapy. *Front Immunol* (2018) 9:9. doi: 10.3389/fimmu.2018.00009

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership