THE THERAPEUTIC POTENTIAL OF ANTIGEN PRESENTING CELLS

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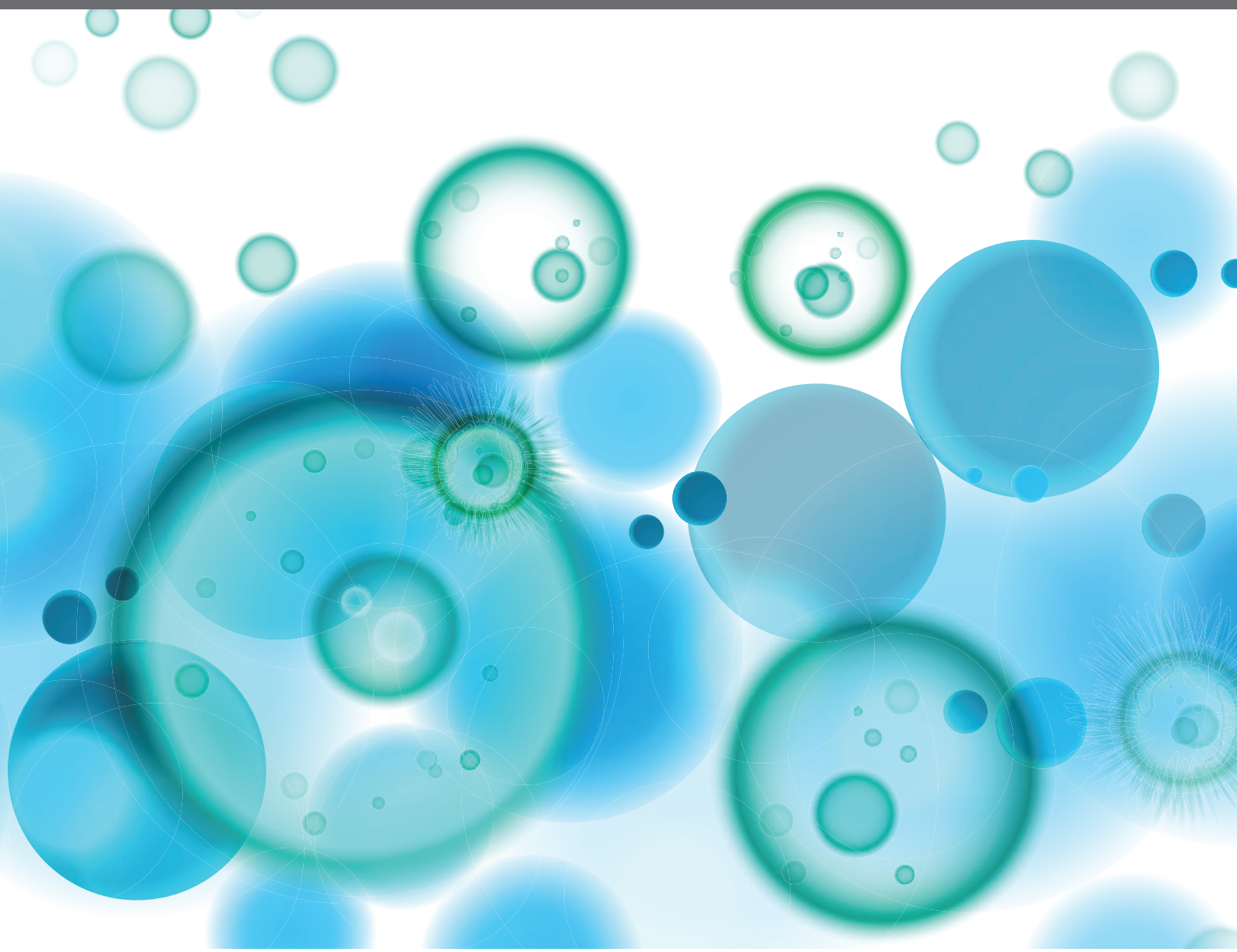

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THE THERAPEUTIC POTENTIAL OF ANTIGEN PRESENTING CELLS

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Development of an Inflammatory CD14$^+$ Dendritic Cell Subset in Humanized Mice

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Humanized mouse models are attractive experimental models for analyzing the development and functions of human dendritic cells (DCs) *in vivo*. Although various types of DC subsets, including DC type 3 (DC3s), have been identified in humans, it remains unclear whether humanized mice can reproduce heterogeneous DC subsets. CD14, classically known as a monocyte/macrophage marker, is reported as an indicator of DC3s. We previously observed that some CD14$^+$ myeloid cells expressed CD1c, a pan marker for *bona fide* conventional DC2 (cDC2s), in humanized mouse models in which human *FLT3L* and *GM-CSF* genes were transiently expressed using *in vivo* transfection (IVT). Here, we aimed to elucidate the identity of CD14$^+$CD1c$^+$ DC-like cells in humanized mouse models. We found that CD14$^+$CD1c$^+$ cells were phenotypically different from cDC2s; CD14$^+$CD1c$^+$ cells expressed CD163 but not CD5, whereas cDC2s expressed CD5 but not CD163. Furthermore, CD14$^+$CD1c$^+$ cells primed and polarized naïve CD4$^+$ T cells toward IFN-γ^+ Th1 cells more profoundly than cDC2s. Transcriptional analysis revealed that CD14$^+$CD1c$^+$ cells expressed several DC3-specific transcripts, such as CD163, S100A8, and S100A9, and were clearly segregated from cDC2s and monocytes. When lipopolysaccharide was administered to the humanized mice, the frequency of CD14$^+$CD1c$^+$ cells producing IL-6 and TNF-α was elevated, indicating a pro-inflammatory signature. Thus, humanized mice are able to sustain development of functional CD14$^+$CD1c$^+$ DCs, which are equivalent to DC3 subset observed in humans, and they could be useful for analyzing the development and function of DC3s *in vivo*.

Keywords: humanized mice, dendritic cell, DC3, CD14, inflammatory response, S100A8, S100A9

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INTRODUCTION

Dendritic cells (DCs) are antigen-presenting cells essential for the innate and acquired immunity (1–3). They comprise various subpopulations that include not only conventional DCs type 1 (cDC1s), type 2 (cDC2s), and plasmacytoid DCs (pDCs) in the systemic compartment, but also monocyte-derived DCs (MoDCs) in the peripheral tissues. These DC subsets have been historically

classified on the basis of their phenotype, functionality, and localization in both humans and mice (4–7). However, differences in phenotypes and functionalities exist in the same DC subsets between humans and mice (8–12). Furthermore, there is a new DC subset in humans, DC3s, whose equivalent counterparts have not yet been identified in mice (13). Thus, it is valuable to establish animal models that precisely reproduce human DC subsets, as they can prove useful for translational research where DCs are utilized for immunotherapy against cancer and infectious diseases (14–16).

Humanized mice, which are reconstituted with human immune cells, are an attractive model for studying differentiated human DCs *in vivo* (17–21). Widely used humanized mouse models have been constructed by transplanting hematopoietic stem cells (HSCs) into severely immunodeficient mice, such as non-obese diabetic (NOD)/SCID/IL2Rγ^{null} (NSG or NOG) mice (22, 23). However, they show poor human DC differentiation and maturation owing to a lack of the responsible cytokines (22, 24). To overcome this limitation, treatment with FMS-like tyrosine kinase 3 ligand (FLT3L) was introduced to improve human DC differentiation in humanized mice (8, 25–27). Recently, another humanized mouse model was established by transplantation of cytokine-expressing stromal cell lines, resulting in improved cDC differentiation (28). However, it remains unclear how precisely humanized mice can reproduce the heterogeneity of DC subsets in humans.

According to the established classification (5), cDCs are defined as distinct subsets from monocytes and macrophages because the DC subsets and monocytes/macrophages are derived from different progenitor cells. However, recent studies revealed that CD1c$^+$ cDCs in human peripheral blood mononuclear cells (PBMCs) comprise heterogeneous subsets, including a subset with monocyte-like characteristics. This subset was originally reported by Villani et al. and named "DC3" (29). DC3s were separated from cDC2s based on the expression of monocyte-related genes, including *CD14, CD163, S100A8*, and *S100A9*, using unbiased transcriptional classification (29). DC3s can be isolated from human PBMCs using DC-related markers and CD14 and/or CD163, and their transcriptional profiles and functionalities are becoming clear (30, 31). In these studies (30, 31), key cytokines (FLT3L and GM-CSF) have been identified for *in vivo* differentiation of DC3s in humans (30) and PBMC-engrafted NSG mice (31). Moreover, the ontogeny and further transcriptional and functional characteristics of DC3s have been discovered using *IRF8* mutated bone marrow and blood samples in humans (32).

Following the report by Villani et al. (29), we previously observed the development of CD14$^+$ DC-like cells along with *bona fide* cDC2s in CD1c$^+$ population in lymphoid tissues of humanized mice (21). Therefore, we aimed to elucidate whether these CD14$^+$CD1c$^+$ cells were equivalent to DC3s and the humanized mice could be used for analyzing DC3 development. In this study, we investigated phenotype, functionality, and transcriptional profiles of CD14$^+$CD1c$^+$ cells compared to those of cDC2s.

MATERIALS AND METHODS

Construction of Humanized Mice

Humanized mice were constructed as described previously (21, 33, 34) using NOD/SCID/Jak3^{null} (NOJ) mice which have an identical phenotype to NSG and NOG mice, with minor modifications. In brief, human HSCs were isolated from umbilical cord blood using the CD133 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated HSCs (1–1.8 $\times$ 10^5 cells) were transplanted into the livers of non-irradiated newborn NOJ mice ($\leq$ 2 days old). Every 4 weeks, starting from 8 week after HSC transplantation, approximately 30 μl of peripheral blood was obtained from the facial vein to determine the extent of chimerism (the percentage of human CD45$^+$ cells within the total peripheral blood cells). Individual humanized NOJ (hNOJ) mice used in this study are listed in **Supplementary Table 1** with information, including the HSC donor ID number, age, and chimerism. Fifteen- to seventeen-week old hNOJ mice were used in this study. All mice were maintained under specific pathogen-free conditions in the animal facility at the National Institute of Infectious Diseases (NIID) (Tokyo, Japan).

In vivo Transfection of Human FLT3L and GM-CSF Using Hydrodynamic Gene Delivery in hNOJ Mice

hNOJ mice at 15–16 weeks post-humanization were subjected to the *in vivo* transfection (IVT) with human *FLT3L* and *GM-CSF* genes to enhance DC development as described previously (21), with minor modifications. In brief, 25 μg of each plasmid DNA encoding human FLT3L and GM-CSF were mixed into TransIT-QR Hydrodynamic Delivery Solution (Mirus, Madison, WI, USA) for hydrodynamic gene delivery. hNOJ mice were intravenously injected with plasmid solution within 4–6 s using a 27-gauge needle. Seven days after IVT, hNOJ mice were sacrificed after the collection of whole blood.

Preparation of Primary Cells From hNOJ Mice and Humans

Mouse primary cells were prepared from whole blood, collected by cardiac puncture, and from the spleen of naïve and IVT-hNOJ mice. Human primary cells were prepared from human peripheral blood of healthy Japanese adult volunteers. Mouse splenocytes were prepared from mouse spleens at 7 days post-IVT using the Spleen Dissociation Kit, mouse (Miltenyi Biotec) and the gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's instructions. For flow cytometry analysis of individual hNOJ mouse samples, dissociated splenocytes were treated with ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA-2Na; pH 7.2–7.4) for 1 min at 25°C to lyse the red blood cells, and then suspended in cold DC-staining buffer (PBS containing 2% heat-inactivated fetal bovine serum, 5 mM EDTA-2Na, and 0.01% sodium azide). For isolation of DCs and monocytes using fluorescence-activated cell sorting (FACS), same HSC donor-derived splenocytes were pooled and subjected to EasySep Mouse/human Chimera Isolation kit

(StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions for the enrichment of human leukocytes. Human T and B cells were depleted from the enriched human leukocytes using the CD3 MicroBead (Miltenyi Biotec) and CD19 MicroBead (Miltenyi Biotec) according to the manufacturer's instructions and then suspended in cold sorting buffer (HBSS containing 2% heat-inactivated fetal bovine serum, 5 mM EDTA-2Na, 25 mM HEPES, 100 μg/ml penicillin, and 100 μg/ml streptomycin) until the cell sorting.

PBMCs from hNOJ mice and PBMCs from healthy human donors were separated using Lymphocyte Separation Medium 1077 (PromoCell, Heidelberg, Germany). Naïve $CD4^+$ T cells from healthy donors' PBMCs were negatively enriched using the EasySep Human Naïve $CD4^+$ T Cell Isolation Kit II (StemCell Technologies) and suspended in IMEM-10 medium [Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% KnockOut Serum Replacement (Thermo Fisher Scientific), 1% GlutaMAX Supplement (Thermo Fisher Scientific), 100 μg/ml penicillin, and 100 μg/ml streptomycin].

Human Leukocytes Flow Cytometry: Staining, Analysis, and Cell Sorting

The fluorochrome-conjugated monoclonal antibodies (mAbs) used are listed in **Table 1**. All mAbs were specific for human antigens. Anti-mouse FcγRII/III (2.4G2) mAb (35) and the Human TruStain FcX (BioLegend), which is compatible with flow cytometric staining with anti-human CD16 (3G8) mAb, were used to prevent non-specific binding of mAbs. LIVE/DEAD Fixable Dead Cell Stain Kits (Aqua, Violet, and Near-IR; Thermo Fisher Scientific) were used for staining dead cells, which were gated out during analysis. For flow cytometric analysis, all cells were incubated with blocking antibodies in DC-staining buffer for 20 min on ice. Then, the samples were washed and stained with a mixture of fluorochrome-conjugated mAbs and with LIVE/DEAD Fixable Dead Cell Kit in DC-staining buffer for 30 min on ice. For cell isolation using FACS, the sorting buffer was used instead of DC-staining buffer. For intracellular staining (ICS), after cell surface staining, cells were fixed and permeabilized using eBioscience Foxp3/Transcription Factor Staining Buffer Set (eBioscience/Thermo Fisher Scientific) according to the manufacturer's instructions. Flow cytometry and cell sorting were performed using BD FACSAria III (BD Biosciences, San Diego, CA, USA). Data were saved as FCS files and analyzed using BD FACSDiva 8.0.1 (BD Biosciences) or FlowJo v10.7.1 (Tree Star/BD Biosciences).

Allogeneic Mixed Lymphocyte Reaction

Naïve $CD4^+$ T cells prepared from human PBMCs were labeled with 5 μM CellTrace Violet (CTV; Thermo Fisher Scientific) according to the manufacturer's instructions. After labeling, cells were washed twice with the IMEM-10 medium. A total of 2,000–2,500 cells from DC subsets or monocytes of hNOJ mice were FACS-sorted into the U-bottom 96-well plate and were subsequently co-cultured with CTV-labeled allogeneic naïve $CD4^+$ T cells at a DC/T cell ratio of 1:20 for 5 days in the IMEM-10 medium at 37°C. On day 5, the $CD4^+$ T cells were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, Mo, USA) and 1 μg/ml ionomycin (Sigma-Aldrich) for 1 h at 37°C. Then, 5 μg/ml Brefeldin A solution was added for 5 h, after which restimulated $CD4^+$ T cells were subjected to the flow cytometry as described above.

Bulk RNA-Sequencing

DC subsets and monocytes from hNOJ mice up to 540 cells isolated using FACS were mixed with 2.7 μl of cold hypotonic lysis buffer consisting of 0.2% (w/v) Triton X-100 (Sigma-Aldrich) and RNase inhibitor (Thermo Fisher Scientific) in 0.2 ml microtubes, immediately frozen on dry ice, and stored at −80°C until cDNA library construction. Cell-lysis solutions were processed to construct cDNA libraries according to the SMART-seq2 protocol (36) with minor modifications. The amplification process was performed with 21 cycles of PCR instead of 18 cycles, and the PCR products were purified using 0.8× volume of AMPure XP beads (Beckman Coulter). When the amount of PCR products was more than 1 ng, they were used for sequencing library preparation using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). The libraries were sequenced with 75 bp paired-end reads on an Illumina Miseq (Illumina).

Bulk RNA-Sequencing Data Processing and Analysis

Adapter sequences and low-quality data were trimmed off from row sequencing reads data of fastq format using flexbar v3.4.0 (37). FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to visualize the read quality. Filtered sequencing reads were aligned to the human reference genome (GRCh38.p13 version 32 release; GENCODE) using HISAT2 v2.1.0 (38) with default parameters. The number of reads assigned to genes was calculated using featureCounts v1.6.4 (39).

Differential gene expression between any pair of samples was assessed using R package DESeq2 v1.28.1 (40), with the default false detection rate adjustment of *p*-values for multiple hypothesis testing. For clustering analysis, raw counts were transformed using variance-stabilizing transformation (VST) (41). Hierarchical clustering was performed using Euclidean distance and complete linkage based on all differentially expressed genes (DEGs) among all cell subsets ($|\mathrm{Log_2FC}| > 1.5$, p-value < 0.01) or 488 genes expressed in at least one sample among cell subsets corresponding to Gene Ontology biological functions of "immune system process" (GO: 0002376) annotated using R package biomaRt v2.28.0 (42), and it was displayed on a heatmap generated using R package pheatmap v1.0.12 (43) following Z-score conversion. A volcano plot displaying DEGs between two subsets ($CD14^+CD1c^+$ cells vs. cDC2s, $CD14^+CD1c^+$ cells vs. monocytes) of hNOJ mice was generated using R package EnhancedVolcano v1.6.0 ($|\mathrm{Log_2FC}| > 1.5$, p-value < 0.01) (44).

For hierarchical clustering using gene expression data from RNA-seq data of hNOJ mice and deposited bulk RNA-seq data of humans, raw sequence data for $CD5^+$ cDC2 [(30), SRA: SRR10056374, SRR10056375, SRR10056376, and SRR10056377],

TABLE 1 | Monoclonal antibodies used for flow cytometry.

Name	Clone	Fluorochrome	Source	Catalog identifier
CD1c	L161	Alexa Flour 700	BioLegend[f]	Cat# 331530, RRID:AB_2563657
CD3	UCHT1	Brilliant Violet 605 PerCP[a]	BioLegend	Cat# 300460, RRID:AB_2564380 Cat# 300427, RRID:AB_893300
CD4	OKT4	Brilliant Violet 605	BioLegend	Cat# 317438, RRID:AB_11218995
CD5	UCHT2	PE[b]	BioLegend	Cat# 300607, RRID:AB_314093
CD8a	RPA-T8	Alexa Flour 700	BioLegend	Cat# 301027, RRID:AB_493744
CD14	RMO52	FITC[c]	Beckman Coulter[g]	Cat# B36297, RRID:AB_130992
CD16	3G8	PerCP	BioLegend	Cat# 302030, RRID:AB_940380
CD19	HIB19	Brilliant Violet 605	BioLegend	Cat# 302244, RRID:AB_2562015
CD33	P67.6	APC-Cy7[d]	BioLegend	Cat# 366614, RRID:AB_2566416
CD45	HI30	Pacific Blue	BioLegend	Cat# 304029, RRID:AB_2174123
CD56	5.1H11	Brilliant Violet 605	BioLegend	Cat# 362538, RRID:AB_2565856
CD88	S5/1	PE	BioLegend	Cat# 344304, RRID:AB_2067175
CD123	6H6	PE-Cy7[e]	BioLegend	Cat# 306010, RRID:AB_493576
CD141	M80	Brilliant Violet 785	BioLegend	Cat# 344116, RRID:AB_2572195
CD163	GHI/61	PE	BioLegend	Cat# 333605, RRID:AB_1134005
CD301/CLEC10A	H037G3	PE	BioLegend	Cat# 354704, RRID:AB_11219002
CD370/CLEC9A	8F9	APC	BioLegend	Cat# 353806, RRID:AB_2565519
IFN-γ	4S.B3	Brilliant Violet 785	BioLegend	Cat# 502541, RRID:AB_11219192
IL-4	8D4-8	PE-Cy7	Thermo Fisher Scientific	Cat# 25-7049-41, RRID:AB_1659722 Cat# 25-7049-82, RRID:AB_469676
IL-6	MQ2-13A5	PE	BioLegend	Cat# 501106, RRID:AB_315154
IL-17A	eBio64DEC17	APC	Thermo Fisher Scientific	Cat# 17-7179-41, RRID:AB_1582221
HLA-DR	L243	PE	BioLegend	Cat# 307605, RRID:AB_314683
S100A8	REA917	PE	Miltenyi Biotec	Cat# 130-115-353, RRID:AB_2727021
S100A9	MRP 1H9	PE	BioLegend	Cat# 350705, RRID:AB_2564007
TNF-α	MAb11	PE	Thermo Fisher Scientific	Cat# 12-7349-81, RRID:AB_466207
Isotype control				
Mouse IgG1 kappa	MOPC-21	APC Brilliant Violet 785 PerCP PE PE-Cy7	BioLegend	Cat# 400120 Cat# 400170 Cat# 400148 Cat# 400112, RRID:AB_2847829 Cat# 400125, RRID:AB_2861433
Mouse IgG2a kappa	MOPC-173	APC PE	BioLegend	Cat# 400222 Cat# 400212, RRID:AB_326460
Rat IgG1 kappa	RTK2071	PE	BioLegend	Cat# 400407, RRID:AB_326513

[a] *Peridinin–chlorophyll protein.*
[b] *Allophycocyanin.*
[c] *Fluorescein isothiocyanate.*
[d] *Allophycocyanin-cyanin 7.*
[e] *Phycoerythrin-cyanin 7.*
[f] *San Diego, CA, USA.*
[g] *Brea, CA, USA.*

DC3 [(31), SRA: SRR11832588, SRR11832589, SRR11832590, and SRR11832591], classical monocyte [(45), cMo; SRA: SRR6298336, SRR6298307, SRR6298370, and SRR6298278], intermediate monocyte [(45), iMo; SRA: SRR6298307, SRR6298308, SRR6298371, and SRR6298279], non-classical monocyte [(45), ncMo; SRA: SRR6298338, SRR6298309, SRR6298372, and SRR6298280], Langerhans cell [(46), LC; SRA: SRR7896371, SRR7896374, and SRR7896377], monocyte-derived macrophage [(47), MDM; SRA: SRR8787287, SRR8787291, and SRR8787295], and MoDC [(48), SRA: SRR6815986, SRR6816010, and SRR6815991] were downloaded from SRA (https://trace.ncbi.nlm.nih.gov/Traces/sra/) using parallel-fastq-dump v0.6.6 (https://github.com/rvalieris/parallel-fastq-dump) and processed as described above. In this hierarchical clustering, the 1,000 most variable genes among all the samples were used.

Gene set enrichment analysis (GSEA; https://www.broad.mit.edu/gsea) (49) was used to assess the expression of

gene signatures specific for two DC subsets of hNOJ mice. Results were considered significant when normalized enrichment score (NES) was over |1.00| and the *q*-value was below 0.25. GSEA was performed using previously published gene signatures defining human blood cDC2s and DC3s listed in **Supplementary Table 2** (29).

Detection of Inflammatory Responses Using *in vivo* ICS Assay

IVT-hNOJ mice were injected intraperitoneally with 200 μl of PBS containing 2 μg of LPS (O55:B5, Sigma-Aldrich) at 7 days post-IVT. One hour after LPS administration, hNOJ mice were intravenously injected with 500 μl of PBS containing 250 μg of Brefeldin A to measure intracellular cytokine synthesis *in vivo* (50, 51). After 5 h, spleens were collected and immediately dissociated, and the splenocytes were stained to detect intracellular cytokines using flow cytometry as described above.

Statistical Analyses

Experimental variables were analyzed using the following statistical tests: the unpaired or ratio-paired *t*-test, Mann-Whitney *U* test, normal or repeated-measures one-way ANOVA followed by the Holm-Sidak's multiple comparison test, and two-way ANOVA followed by the Holm-Sidak's multiple comparison test (see individual figure legends). GraphPad Prism software version 6 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. A p-value < 0.05 was considered statistically significant.

RESULTS

$CD14^+CD1c^+$ Cells Are Phenotypically Similar to DC3

Previously, we established a humanized mouse model that enabled the enhanced development of human DC subsets using IVT of human *FLT3L* and *GM-CSF* genes (21). This mouse model was used in this study. The gating strategy for each DC(-like) subset was the same as that in the previous study, except for the following one point (**Figure 1A** and **Supplementary Figures 1A,B**). To reduce the number of false-positive cells because of spillover of other fluorochromes, we used an anti-CD14 mAb conjugated with FITC, instead of ECD. We first selected human $CD45^+CD3^-CD19^-CD56^-CD33^+$ splenocytes in hNOJ mice, and then subdivided these cells into three populations: $CD141^+$ population (cDC1), $CD1c^+$ population (cDC2), and $CD1c^-CD141^-$ (DN) population (**Supplementary Figure 1A**). CD14 expression in the three populations was comparatively plotted based on the gating threshold that was set by the isotype control staining (**Figure 1A** and **Supplementary Figure 1B**). cDC1 population was negative for CD14 expression, and DN population expressed CD14 at various levels (**Figure 1A**). For human PBMCs, the DN population harbored classical monocyte populations that exhibited $CD14^{high}CD16^-$ phenotype (**Supplementary Figure 1C**). When $CD14^+$ cells in DN populations of hNOJ mice were divided into $CD14^{high}$ cells and $CD14^{low}$ cells, $CD14^{high}$ DN cells highly expressed CD88, a recently defined marker for the monocyte (30, 31), however, $CD14^{low}$ DN cells exhibited heterogeneous CD88 expression (**Supplementary Figure 1D**). Taken together, we gated a $CD14^{high}CD16^-$ fraction in DN population of hNOJ mice as a putative classical monocyte subset. In addition to these expected results, we noticed the presence of a small $CD14^+$ fraction within the cDC2 population (∼1% within the $CD1c^+$ population) of hNOJ mice (**Figure 1A**). $CD14^+$ cells were detectable in all humanized mice ($n = 15$), but the frequencies were 150-fold less than those of $CD14^-CD1c^+$ cDC2 cells (**Figures 1B,C**). When human PBMCs were analyzed by the same gating strategy, $CD14^+$ cells were also found in $CD1c^+$ population with $CD14^{high}$ and $CD14^{low}$ population (**Supplementary Figure 2A**). $CD14^{low}CD1c^+$ cells and $CD14^-CD1c^+$ cells (cDC2s) in human PBMCs were negative for CD88 (**Supplementary Figure 2B**). Moreover, these $CD14^{low}CD1c^+$ cells highly expressed CD163 (**Supplementary Figure 2C**), suggesting that our gating strategy indeed identifies human DC3 subset as $CD14^{low}CD1c^+$ cells. Then, we compared the ratios of $CD14^+CD1c^+$ cells vs. cDC2s between splenocytes in hNOJ mice and human PBMCs, and found that they were approximately seven times higher in human PBMCs than in splenocytes of hNOJ mice (**Supplementary Figure 2D**). Nevertheless, this atypical cellular subset, as well as cDC1s and cDC2s, was found at increased frequencies in IVT-hNOJ mice compared to untreated hNOJ mice (**Supplementary Figure 2E**). Thus, we identified $CD14^+CD1c^+$ cells that were induced in IVT-hNOJ mice at elevated frequencies.

Next, we examined the expression of other DC- and monocyte/macrophage-related surface markers (CLEC9A, CLEC10A, HLA-DR, CD163, CD5, and CD88) on these subsets in the spleens of hNOJ mice (**Figure 1D**). CLEC9A and CLEC10A were selectively expressed in cDC1 and cDC2, respectively, as expected since they have been previously utilized as cDC1 and cDC2 markers (52, 53). Interestingly, $CD14^+CD1c^+$ cells expressed cDC2 marker CLEC10A at frequencies similar to cDC2s, but $CD14^+$ monocyte-like cells did not. We verified the expression of HLA-DR in all four subsets in hNOJ mice, in corroboration with the results of the previous study (54), and HLA-DR level on $CD14^+CD1c^+$ cells was closer to that observed on cDC2s than to the HLA-DR level observed on monocytes. Thus, our CLEC10A and HLA-DR expression data support a similarity between $CD14^+CD1c^+$ cells and cDC2s.

Recently, a new DC subset was identified that is phenotypically different from cDC1 and cDC2; therefore, it was denominated DC3 (29). To further characterize $CD14^+CD1c^+$ cells, we examined the surface expression of other molecules that are differentially expressed among cDC1, cDC2, and DC3. CD163, a phenotypic marker for DC3 (29, 30, 55), was selectively expressed on $CD14^+CD1c^+$ cells, although the frequency of $CD163^+$ cells varied depending on the HSC donor (**Figure 1E**). In contrast, the cDC2-related marker, CD5 (56), was selectively expressed on cDC2, but not on $CD14^+CD1c^+$ cells. The monocyte marker, CD88, was highly expressed on monocytes of hNOJ mice. In $CD14^+CD1c^+$ cells, some cells expressed CD88,

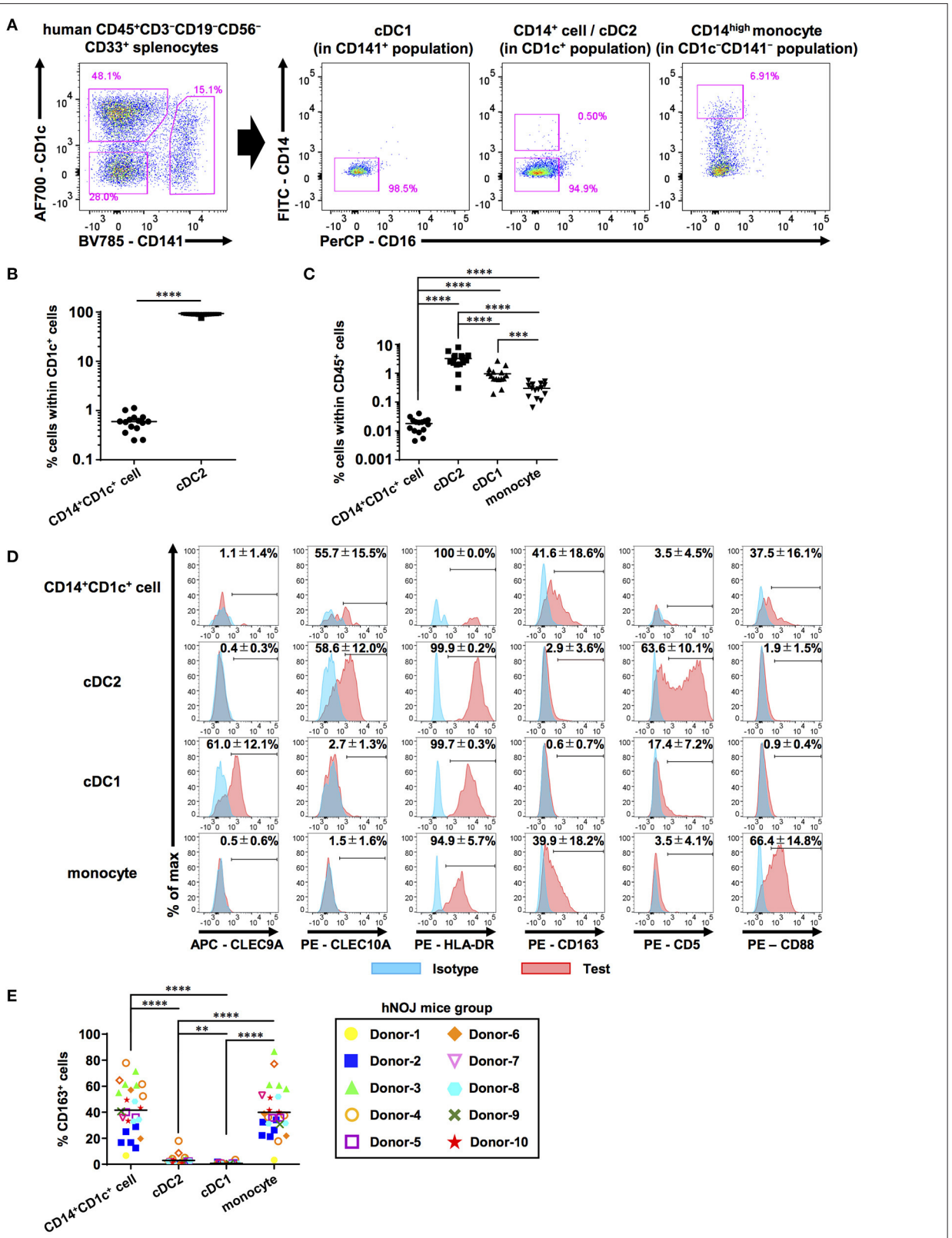

FIGURE 1 | Characterization of human DC populations in hNOJ mice. Cells were prepared from the spleen of humanized NOJ (hNOJ) mice following *in vivo* transfection (IVT). **(A)** A representative gating strategy for cDC1s, cDC2s, $CD14^{+}CD1c^{+}$ cells, and $CD14^{high}$ monocytes. **(B)** Individual percentages of $CD14^{+}CD1c^{+}$

(Continued)

FIGURE 1 | cells and cDC2s within CD1c$^+$ population ($n = 15$). A significant difference (****$P < 0.0001$) was determined using the ratio-paired t-test. **(C)** Individual percentages of CD14$^+$CD1c$^+$ cells, cDC2s, cDC1s, and monocytes within human CD45$^+$ cells ($n = 15$). Significant differences (***$P < 0.001$, ****$P < 0.0001$) were determined using the repeated-measures one-way ANOVA followed by the Holm-Sidak's multiple comparison test. **(D)** Representative histogram profiles for subset-associated markers on CD14$^+$CD1c$^+$ cells, cDC2s, cDC1s, and monocytes using flow cytometry (red: test marker staining, blue: isotype staining). The percentages in each panel show the mean ± SD of marker positive cells in each population (CLEC9A: $n = 8$, CLEC10A: $n = 4$, HLA-DR: $n = 4$, CD163: $n = 26$, CD5: $n = 5$, CD88: $n = 8$). **(E)** Individual percentages of CD163$^+$ cells within each cell population related to **(D)**. The same color-symbols show the same donor-derived hNOJ mice. Significant differences (**$P < 0.01$, ****$P < 0.0001$) were determined using the repeated-measures one-way ANOVA followed by the Holm-Sidak's multiple comparison test.

but its expression was lower than monocytes like the CD14 expression pattern. Taken together, these results indicate that the phenotype of CD14$^+$CD1c$^+$ cells from hNOJ mice is highly similar to human DC3 phenotype.

CD14$^+$CD1c$^+$ Cells Are Functionally Competent for Priming and Polarizing Naïve CD4$^+$ T Cells

We addressed whether CD14$^+$CD1c$^+$ cells can activate naïve CD4$^+$ T cells. First, we performed an allogeneic mixed lymphocyte reaction to evaluate CD4$^+$ T-cell priming capabilities of these populations. CD14$^+$CD1c$^+$ cells, cDCs, and monocytes were isolated and co-cultured with CTV-labeled allogeneic naïve CD4$^+$ T cells derived from human PBMCs. After co-culture for 5 days, the proliferation of CD4$^+$ T cells was monitored by CTV degradation using flow cytometry (**Figures 2A,B**). Co-cultures of CD14$^+$CD1c$^+$ cells, cDC2s, and cDC1s induced proliferation of naïve CD4$^+$ T cells, whereas co-culture of monocytes induced little proliferation of naïve CD4$^+$ T cells at the level similar to the culture condition of CD4$^+$ T cells alone (**Figure 2C**).

After T-cell priming, DCs polarize naïve CD4$^+$ T cells into divergent T-cell subsets depending on DC functions (57). Therefore, it is important to clarify which types of T cell subsets are induced following interaction with individual DC subsets. After co-culture for 5 days with either CD14$^+$CD1c$^+$ cells, cDC2s, or cDC1s, CD4$^+$ T cells were restimulated with PMA and ionomycin for 6 h. Then, we evaluated the frequencies of proliferated CD4$^+$ T cells (CTV^{low}CD4$^+$ T cells) that produced Th1 and Th2 cytokines (IFN-γ and IL-4, respectively) using ICS (**Figure 2D**, **Supplementary Figure 3A**). We first examined the frequency of these CD4$^+$ T cells within total CD4$^+$ T cells to evaluate how much the co-cultured DC subset polarized naive CD4$^+$ T cells. However, since the degree of cell proliferation among samples varied greatly, there is no significant difference in the amount of polarized CD4$^+$ T cells (**Supplementary Figure 3B**). Therefore, to accurately characterize the induced polarization, we focused only on proliferated CD4$^+$ T cells and compared the frequency of cytokine-producing cells, and observed that CD14$^+$CD1c$^+$ cells have greater Th1-polarizing capacity compared with cDC2 (**Figure 2E**). These results indicate that CD14$^+$CD1c$^+$ cells can be discriminated from cDC2s by their functional aspects. Of note, a previous study has shown similar Th1-polarizing capacity in DC3s of human PBMCs (31). Thus, the Th1-polarizing capacity, in addition to phenotypic markers, highlights the similarity between CD14$^+$CD1c$^+$ cells and DC3s. We also evaluated Th17-polarizing capacity based on IL-17A expression, another characteristic of DC3s (30). However, we did not observe IL-17A$^+$CD4$^+$ T cells within CD4$^+$ T cells that were stimulated with CD14$^+$CD1c$^+$ cells, cDC1s, and cDC2s (**Supplementary Figures 3C,D**). Collectively, our data demonstrate a functional similarity between CD14$^+$CD1c$^+$ cells from hNOJ mice and DC3s from human PBMCs.

Transcriptional Profile Reveals DC3-Specific and Inflammatory Signatures in CD14$^+$CD1c$^+$ Cells

We characterized transcriptional profiles of CD14$^+$CD1c$^+$ cells and compared them with those of cDC2s and monocytes. CD14$^+$CD1c$^+$ cells, cDC2s, and monocytes were isolated from splenocytes pooled from same donor HSC-derived hNOJ mice. Three mouse groups from different donors were used. When RNA-seq analysis was carried out, one analysis from monocytes was excluded because cDNA amplification was insufficient. First, to elucidate whether CD14$^+$CD1c$^+$ cells were transcriptionally profiled as a population closer to the cDC2 population or to the monocyte population, we performed the hierarchical clustering using all DEGs among all cell subsets (**Figure 3A**; $|\mathrm{Log_2FC}| > 1.5$, p-value < 0.01). Three clusters of each cell subset were formed, and the cluster of CD14$^+$CD1c$^+$ cells was found to be closer to cDC2s than to monocytes. We also performed the hierarchical clustering of 488 genes, which are annotated in Gene Ontology database as contributing to "immune system process" and expressed in at least one sample (**Figure 3B**). Similar to the result of clustering using DEGs, the cluster of CD14$^+$CD1c$^+$ cells was closer to cDC2s than monocytes. Interestingly, both groups of genes upregulated in cDC2 clusters and monocyte clusters tended to be upregulated in clusters of CD14$^+$CD1c$^+$ cells (**Figure 3A**), suggesting that CD14$^+$CD1c$^+$ cells have both transcriptional characteristics of cDC2 and monocyte. Therefore, we compared the similarity of CD14$^+$CD1c$^+$ cells of hNOJ mice to the human CD1c$^+$ DC subsets or monocyte-related subset using public data. The results showed that CD14$^+$CD1c$^+$ cells were closest to cDC2 of hNOJ mice, followed by human-derived cDC2 and DC3 clusters (**Supplementary Figure 4**).

To further characterize CD14$^+$CD1c$^+$ cells, we identified DEGs between CD14$^+$CD1c$^+$ cells vs. cDC2s and CD14$^+$CD1c$^+$ cells vs. monocytes in hNOJ mice ($|\mathrm{Log_2FC}| > 1.5$, p-value < 0.01). All DEGs were listed in **Supplementary Table 3**, and genes in this table that have been previously reported to be characteristic of human DC3, cDC2, and monocyte (29, 31, 32) were selected and labeled in the volcano plot

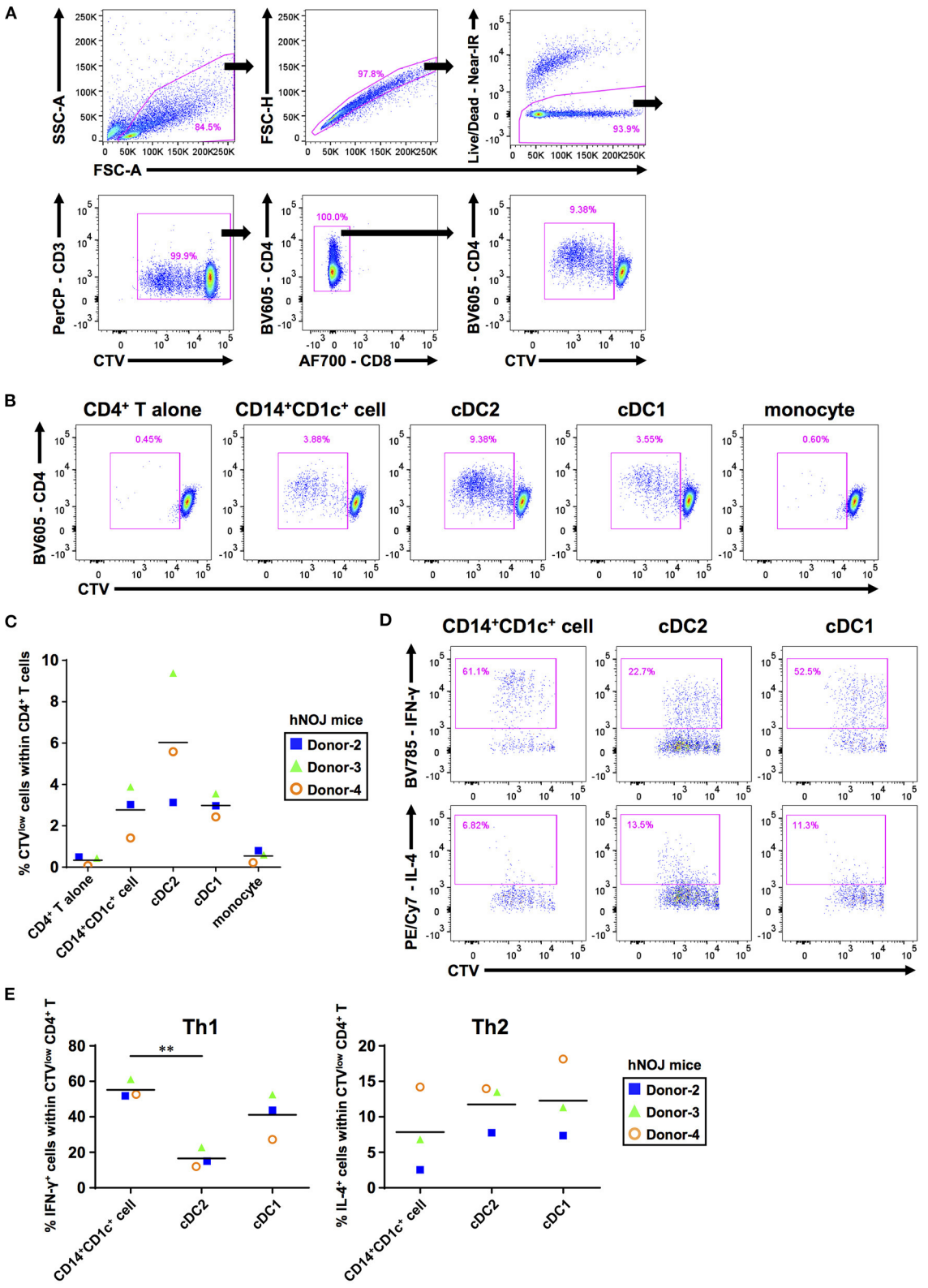

FIGURE 2 | The ability to prime and polarize Th cells among human dendritic cell populations derived from hNOJ mice. DCs and monocytes were isolated from pooled spleens of donor-matched hNOJ mice following IVT, and $CD4^+$ T cells were prepared from allogeneic human peripheral blood mononuclear cells. $CD4^+$ T cells

(Continued)

FIGURE 2 | were subjected to flow cytometric analysis following co-culture for 5 days with DCs or monocytes. **(A)** A representative gating strategy for $CD4^+$ T-cell proliferation. **(B)** Representative flow cytometry profiles of $CD4^+$ T-cell proliferation based on the CellTrace Violet (CTV) intensity in each co-culture condition. **(C)** Individual percentages of CTV^{low} cells within $CD4^+$ T cells. The same color symbols show the same donor-derived hNOJ mice. The repeated-measures one-way ANOVA followed by the Holm-Sidak's multiple comparison test was used to compare among co-culture conditions, and no significant differences were observed. **(D)** Representative flow cytometry profiles of IFN-γ *and* IL-4 expression in $CD4^+$ T cells. **(E)** Individual percentages of IFN-γ^+ cells (Th1; $n = 3$) and IL-4^+ cells (Th2; $n = 3$) within $CTV^{low}CD4^+$ T cells. The same color symbols show the same donor-derived hNOJ mice. Significant differences (**$P < 0.01$) were determined using the repeated-measures one-way ANOVA followed by the Holm-Sidak's multiple comparison test.

(**Figure 3C**). In a comparison of $CD14^+CD1c^+$ cells and cDC2 of hNOJ mice, $CD14^+CD1c^+$ cells highly expressed *CD14* and *CD163*, whereas cDC2s expressed *CD1c* and *CD5*. These transcriptional data were consistent with the surface expression profiles shown in **Figure 1**. In addition, we observed the upregulation of *S100A8*, *S100A9*, and *IL-6* in $CD14^+CD1c^+$ cells, which are related to inflammation (58). Moreover, $CD14^+CD1c^+$ cells exhibited higher expression of DC3-related genes, including *S100A12*, *TMEM176A,* and *TMEM176B* (29, 31). In a comparison of $CD14^+CD1c^+$ cells and monocytes of hNOJ mice, similar to previous reports of DEGs in human DC3s and monocytes (31, 32), $CD14^+CD1c^+$ cells highly expressed *CD1c, CLEC10A, GPR183, HLA-DQA1* and *HLA-DPB1*, while monocytes highly expressed *S100A8, S100A9, S100A12, FCAR, TLR4* and *VCAN*.

GSEA clarifies any enrichment of specific gene signatures in a pairwise comparison of gene expression data derived from two cell populations (49); therefore, it was applied to determine the similarity between $CD14^+CD1c^+$ cells from hNOJ mice and DC3s from human PBMCs. We used published gene signatures of DC3s (DC3 > ALL and DC3 > cDC2) and cDC2s (cDC2 > ALL and cDC2 > DC3) (**Supplementary Table 2**), according to Villani et al. where DC3/cDC2 was compared with other DC subsets in which $CD14^+$ cells were excluded (29). DC3 gene signatures (DC3 > ALL and DC3 > cDC2) were significantly enriched in $CD14^+CD1c^+$ cells (**Figure 3D**; DC3 > ALL: NES = 1.42, $q = 0.027$; DC3 > DC2: NES = 1.74, $q < 0.001$), whereas a cDC2 gene signatures (cDC2 > DC3) were significantly enriched in cDC2s from hNOJ mice (**Figure 3D**; NES = −1.49, $q = 0.026$). Therefore, these results indicate that $CD14^+CD1c^+$ cells from hNOJ mice are a subset closely related to DC3s from human PBMCs, based on transcriptional profiles.

$CD14^+CD1c^+$ Cells Express Pro-inflammatory Mediators by LPS Administration

Since transcriptional analysis revealed the inflammatory signatures of $CD14^+CD1c^+$ cells, we next examined whether $CD14^+CD1c^+$ cells exert a pro-inflammatory response in acute inflammation.

When LPS was administrated to hNOJ mice to produce acute inflammation, we observed that LPS had little effect on the absolute numbers of all myeloid cell subsets ($CD14^+CD1c^+$ cells, cDC1s, cDC2s, and monocytes), although there was a large variation among individual mice (**Figure 4A**).

Then, we analyzed the expression of two intracellular inflammation-related proteins, namely S100A8 and S100A9 (also known together as "calprotectin"), and of two cytokines, namely IL-6 and TNF-α, in each cell subset using flow cytometry and compared their levels between steady (non-LPS-stimulated) state and acute inflammatory state (**Figure 4B**). The gating threshold of $S100A8^+$ and $S100A9^+$ cells was set with reference to the flow cytometry profiles of cDC2, which does not express S100A8 and S100A9 (31). The gating threshold of IL-6^+ and TNF-α^+ cells was set by the isotype control staining (**Supplementary Figures 5A,B**). As a result, we found that most $CD14^+CD1c^+$ cells and monocytes, but not cDC1s and cDC2s, expressed S100A8 and S100A9, regardless of LPS administration (**Figures 4C,D**). In addition, the frequencies of IL-6 producing cells were significantly higher in $CD14^+CD1c^+$ cells than in all subsets except for cDC2s in steady state (**Figure 4E**). After LPS injection, the frequencies of IL-6 producing cells were elevated in all subsets except for cDC1s, but $CD14^+CD1c^+$ cells reached the highest numbers of IL-6-producing cells among all subsets from the same mice (**Figure 4E**). Similar to DC3s (31), we also found TNF-α expression in $CD14^+CD1c^+$ cells after LPS injection (**Figure 4F**). Collectively, our data show that $CD14^+CD1c^+$ cells constitutively produce inflammatory-related calprotectin and upregulate pro-inflammatory cytokines under acute inflammatory conditions in humanized mice.

DISCUSSION

Since DC3s have been proposed as a DC subpopulation distinct from cDC2s (29), the functional and transcriptional profiles of DC3s have been investigated in several studies using human peripheral blood (30–32). However, tissue-resident DC3s remain uncharacterized owing to limited access to tissue cells. Therefore, it is desirable to establish a small animal model that is able to reproduce the development of DC3s. Here, we have identified inflammatory $CD14^+CD1c^+$ DCs in humanized mice as the equivalent to DC3s in humans.

In this study, we investigated whether humanized mice are able to develop a DC subpopulation similar to human DC3s by focusing on the identity of $CD14^+$ cells within the $CD1c^+$ cell population. To examine this, we used our previously established humanized mouse model in which human FLT3L and GM-CSF were expressed by using an IVT strategy (21). In our previous study (21), expression of human FLT3L and GM-CSF enhanced cDC1 and cDC2 development. This study shows that it also promoted $CD14^+CD1c^+$ cell development

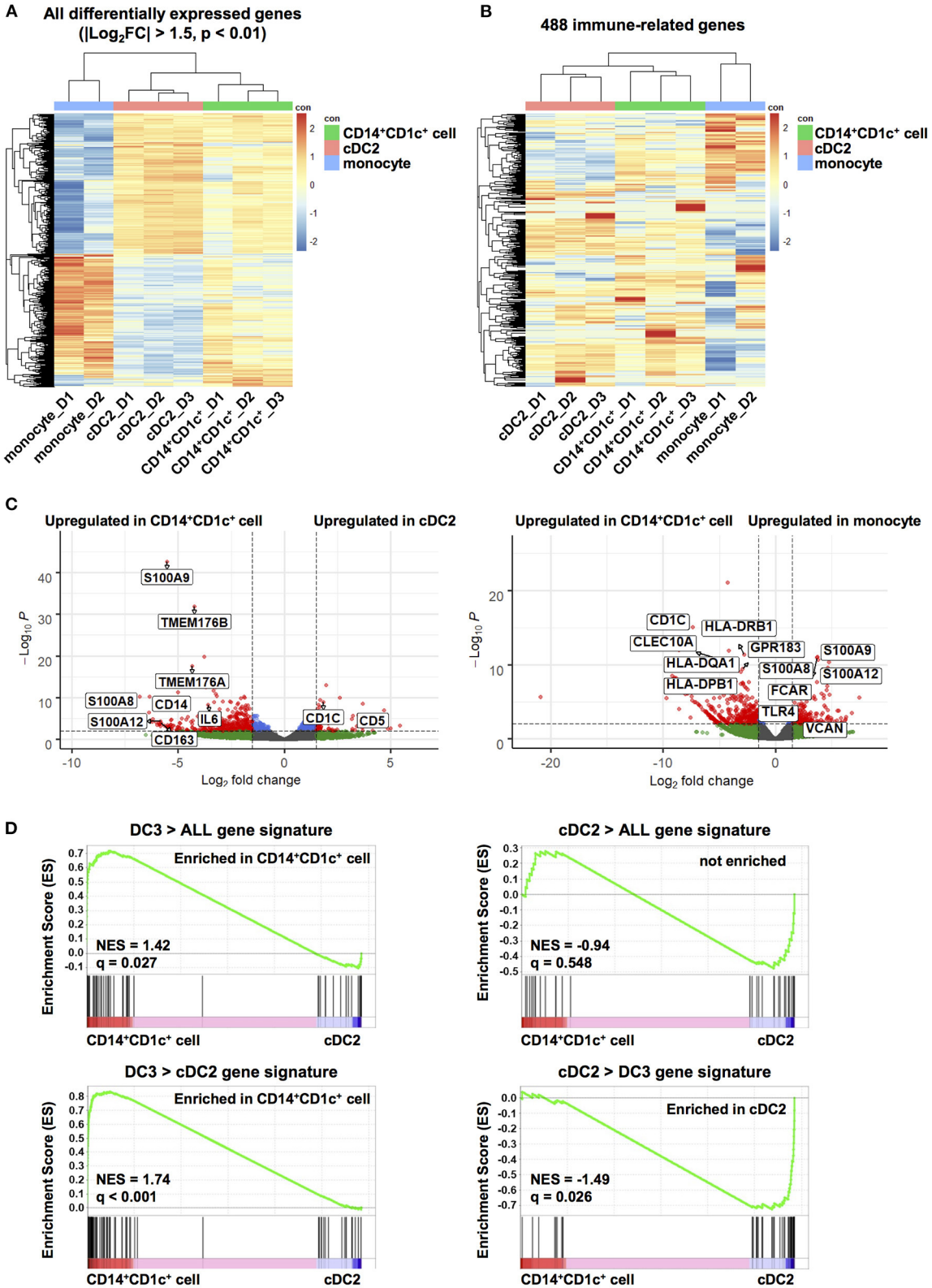

FIGURE 3 | Transcriptome analysis of human dendritic cell populations in hNOJ mice. Total RNA was extracted from $CD14^+CD1c^+$ cells, cDC2s, and monocytes isolated from the spleen of IVT-hNOJ mice and sequenced. **(A)** Heatmap visualization of the z-scores for the all DEGs among $CD14^+CD1c^+$ cells ($n = 3$), cDC2s

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FIGURE 3 | ($n = 3$), and monocytes ($n = 2$) obtained ($|Log_2FC| > 1.5$, p-value < 0.01) using the hierarchical clustering analysis. **(B)** Heatmap visualization of the z-scores for 488 genes expressed in at least one sample among $CD14^+CD1c^+$ cells ($n = 3$), cDC2s ($n = 3$), and monocytes ($n = 2$) obtained using the hierarchical clustering analysis using with the sets of genes corresponding to Gene Ontology annotation "immune system process." The colors in the heatmap indicate high (red) or low (blue) expression across the sample set. **(C)** Volcano plots displaying DEGs between two subsets in hNOJ mice ($CD14^+CD1c^+$ cells vs. cDC2s, $CD14^+CD1c^+$ cells vs. monocytes). Genes with $|Log_2(\text{fold change})| > 1.5$, and p-value < 0.01 were considered significant (red plot). **(D)** GSEA comparing $CD14^+CD1c^+$ cells and cDC2s derived from hNOJ mice using published cDC2s and DC3s gene signatures (29). Statistical significance was defined by normalized enrichment score (NES) and q-value calculated with GSEA software using the default parameter.

(**Supplementary Figure 2E**). Recent reports regarding the ontogeny of DC3s indicate that enhanced differentiation of DC3s can be achieved through administration of FLT3L and/or GM-CSF (30, 31). Therefore, we considered our FLT3L- and GM-CSF-IVT strategy reasonable to investigate the development of DC3s in humanized mice. However, in this study, we encountered difficulties in analyzing all DC subsets in the peripheral blood of hNOJ mice because of low yield, notwithstanding FLT3L and GM-CSF expression. Because of this limitation, we were unable to compare the $CD14^+CD1c^+$ cells in the spleen with those in the blood. It will be necessary to clarify whether the DC3-like $CD14^+CD1c^+$ cells observed in the spleen are differentiated *in situ* from the progenitor or have entered the spleen from the blood.

The $CD1c^+$ population from hNOJ mice consisted of heterogeneous DCs with differential expression of CD5, CD14, and CD163 (**Figure 1D**), similar to the $CD1c^+$ population from human peripheral blood (29, 30, 56). In general, during flow cytometric analysis, $CD14^+$ cells are first gated out, in order to separate cDCs from $CD14^+$ monocytes. In contrast, we first focused on the DC-like population with cDC-specific markers, followed by fractionation of DCs depending on the CD14 expression level (21). This gating strategy allowed us to capture DC3-like $CD14^+CD1c^+$ cells from a heterogeneous $CD1c^+$ population (**Figures 1A,B**). Recent studies analyzing DC3s isolated from human PBMCs also adopted similar gating strategies, in which CD14-positive cells were not excluded (30, 31). Although these studies used a different set of markers to identify DC3s (e.g., CD5, CD163, BTLA), they consistently used CD14 marker as one of the selecting markers for DC3s. These studies showed much larger DC3 populations (DC3:cDC2 = 1:2 to 1:6) in human PBMCs (30, 31) than our $CD14^+CD1c^+$ cell population ($CD14^+CD1c^+$ cell:cDC2 = 1:150) in the spleen of hNOJ mice (**Figure 1B**). When we analyzed human PBMCs using the same gating strategy as the analysis for hNOJ mice (**Supplementary Figure 2A**), the ratio of $CD14^{low}CD1c^+$ cells to cDC2s was 1:20 and was lower than the previous studies (30, 31), possibly due to different gating strategies. However, the ratio was still higher than that of hNOJ mice (**Supplementary Figure 2D**). This difference in the ratio of DC3 ($CD14^+CD1c^+$ cell population) to cDC2 between human and hNOJ mice may be due to the difference in developmental conditions within humanized mouse models and humans, including the lack of all human cytokines other than FLT3L and GM-CSF in hNOJ mice. $CD14^+CD1c^+$ cells found in humanized mice may not fully represent *bona fide* DC3s because of different human cytokine milieu and low expression of CD88 in some $CD14^+CD1c^+$ cells. However, our identification of a DC subset distinct from cDC2s, on the basis of CD14 expression level, may be an important finding toward unifying the fractionation of DC3s, which still varies among research groups.

In human myeloid cells, CD14 and CD1c double-positive cells include not only DC3s but also monocyte-derived macrophages and MoDCs (30, 59–61). Unlike macrophages, DCs are known to be fully capable of activating naïve T cells (61, 62). We showed that $CD14^+CD1c^+$ cells as well as other cDCs, primed and promoted the proliferation of naïve $CD4^+$ T cells at higher levels than did monocytes (**Figure 2C**), supporting a distinct functionality of $CD14^+CD1c^+$ cells from macrophages. Moreover, it is difficult to distinguish between DC3s and MoDCs because their functionality and transcription profiles tend to overlap conspicuously and no discriminative markers have been reported yet (13). However, our transcriptional analysis demonstrated that $CD14^+CD1c^+$ cells isolated from hNOJ mice are closer to cDC2s and DC3s than to MoDCs in humans (**Supplementary Figure 4**). In addition, the development of inflammatory MoDC is believed to require IL-4 (63, 64), and the development of $CD14^+CD1c^+$ cells in hNOJ mice was enhanced in the absence of human IL-4 (**Supplementary Figure 2E**). These results indicated that $CD14^+CD1c^+$ cells isolated from hNOJ mice could be discriminated from monocyte-derived macrophages and MoDCs.

Recent studies on DC3s share a common understanding that $CD14^+$ DC3s, as well as cDC2s, stimulate and induce proliferation of naïve T cells (29–31). Bourdely et al. (31) showed that naïve T cells could be polarized into Th1 cells, whereas Dutertre et al. (30) showed that they could be significantly polarized into Th17 cells but not into Th1 cells. This difference may depend on the activation status of DCs: the former study used DC3s that was activated using multiple TLR ligands after isolation (31), and the latter study used unstimulated DC3s after isolation (30). In the present study, we demonstrated that $CD14^+CD1c^+$ cells polarized Th1 cells but not Th17 cells (**Figure 2E** and **Supplementary Figure 3D**), corroborating with the results of the study by Bourdely et al. (31) regarding the properties of DC3s. Since we previously observed that IVT of hNOJ mice with FLT3L- and GM-CSF-encoding plasmids could enhance the activation/maturation of cDCs (21), it is likely that activated $CD14^+CD1c^+$ cells may show similar properties to DC3s as reported by Bourdely et al. (31).

Although $CD14^+CD1c^+$ cells did not polarize Th17 cells, $CD14^+CD1c^+$ cells markedly produced pro-inflammatory cytokines IL-6 and TNF-α in LPS-induced acute inflammation (**Figures 4E,F**). These pro-inflammatory signatures were similar to DC3s, which have been reported to be more potent producers of pro-inflammatory cytokines (namely IL-1β,

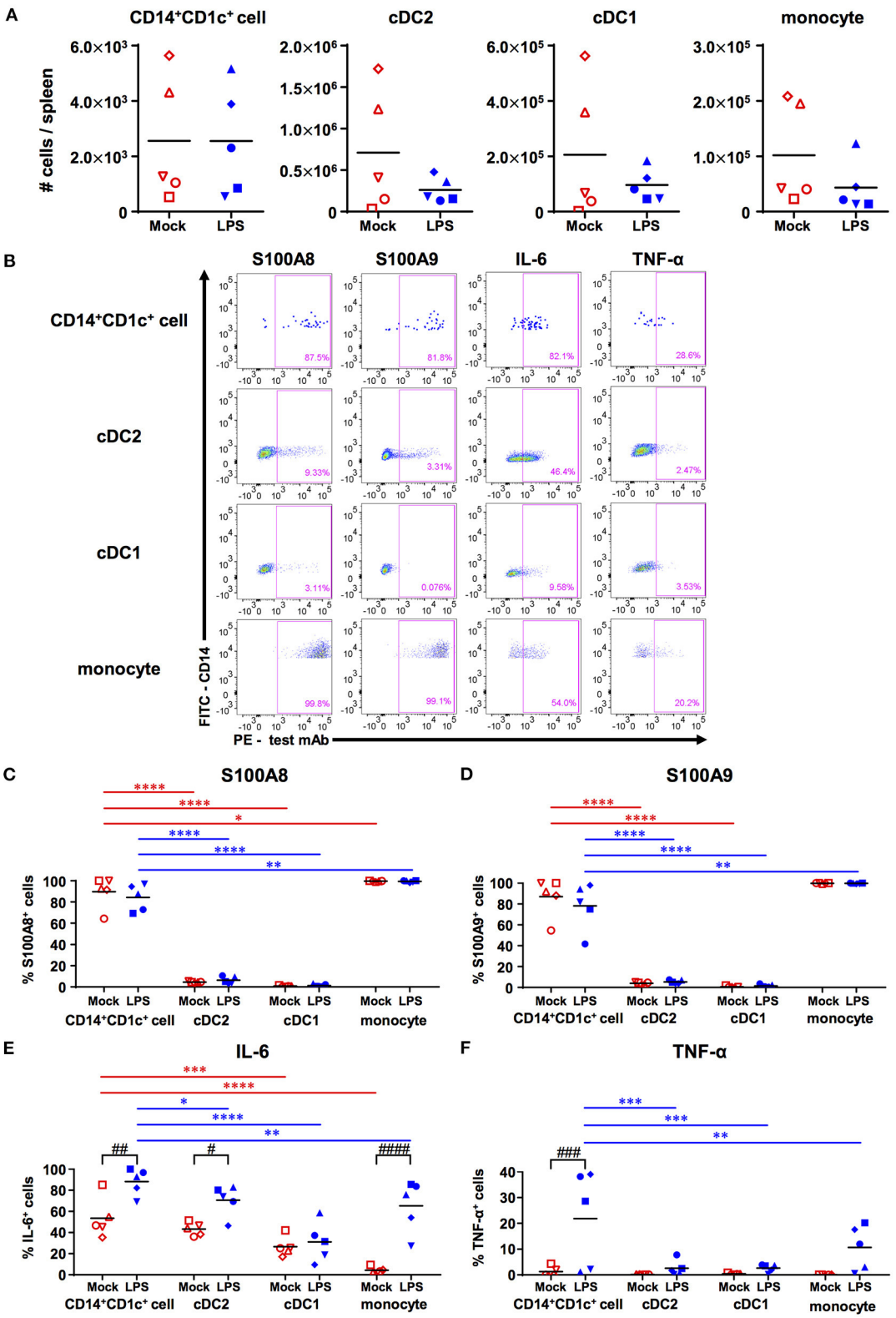

FIGURE 4 | Responsiveness of human dendritic cell populations in hNOJ mice against LPS-induced acute inflammation. Cells were prepared from the spleen of IVT-hNOJ mice following intraperitoneal LPS injection. **(A)** Individual absolute cell numbers of $CD14^+CD1c^+$ cells, cDC2s, cDC1s, and monocytes within human

(Continued)

FIGURE 4 | $CD45^+$ cells (Mock: $n = 5$, LPS: $n = 5$). The Mann-Whitney *U* test was used, and no significant differences were observed. The distinct symbols show each separate hNOJ mouse. **(B)** Representative flow cytometry profiles of intracellular inflammation-related proteins/cytokines (S100A8, S100A9, IL-6, and TNF-α) in cell populations. **(C–F)** Individual percentages of the cells expressing **(C)** S100A8, **(D)** S100A9, **(E)** IL-6, and **(F)** TNF-α (Mock: $n = 5$, LPS: $n = 5$). Significant differences ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$) between $CD14^+CD1c^+$ cells and each other subset under the same conditions (red: mock, blue: LPS) were determined using the repeated-measures two-way ANOVA followed by the Holm-Sidak's multiple comparison test. In addition, significant differences ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, and $^{\#\#\#\#}P < 0.0001$) between Mock and LPS conditions in same cell subsets were determined by repeated-measures two-way ANOVA followed by the Holm-Sidak's multiple comparison test. The distinct symbols show each separate hNOJ mouse.

IL-8, and TNF-α) than cDC2s under various stimulated conditions (30–32). In addition, most of $CD14^+CD1c^+$ cells constitutively produced S100A8 and S100A9, regardless of the LPS administration (**Figures 4C,D**). S100A8 and S100A9 are collectively known as calprotectin, mainly produced by neutrophils and monocytes/macrophages in response to inflammation by autoimmunity or infection (58, 65). Moreover, recent studies using RNA-seq analysis have shown that mRNA of S100A8 and S100A9 is constitutively expressed in DC3s derived from healthy donors (29–31). These results suggest that $CD14^+CD1c^+$ cells are a potent pro-inflammatory DC subset, like DC3s.

The gene signature adopted for DC3s in the transcriptional analysis in recent studies (30, 31), including our present one, is based on the results of Villani et al. (29). Our bulk RNA-seq performed on $CD14^+CD1c^+$ cells showed a gene profile close to *bona fide* DC3s (**Figure 3D**), because gene signatures of DC3s (DC3 > cDC2 and DC3 > ALL) reported by Villani et al. (29) were enriched in $CD14^+CD1c^+$ cells. However, the gene signature of cDC2s (cDC2 > ALL) (29) overlapped in $CD14^+CD1c^+$ cells and could not clearly distinguish between $CD14^+CD1c^+$ cells and cDC2s of hNOJ mice (**Figure 3D**). In addition, although Cytlak et al. reported that DC3s expressed higher levels of IL-1β than cDC2 (32), our RNA-Seq results showed that the expression levels of IL-1β transcripts were comparable between $CD14^+CD1c^+$ cells and cDC2, resulting in unidentified *IL-1β* in DEGs (**Supplementary Table 3**). The discrepancy would be due to the different cell isolation/separation strategy, e.g., we isolated $CD14^+$ cells from $CD1c^+$ cells as a DC3 counterpart, whereas Villani et al. (29) separated between DC3s and cDC2s among $CD14^-CD1c^+$ cells by scRNA-seq analysis. Indeed, $CD14^+CD1c^+$ cells in hNOJ mice exhibited heterogeneous surface CD163 expression (**Figure 1E**), whereas *bona fide* DC3s and cDC2s in human PBMCs were clearly distinguished as CD163-positive cells and CD163-negative cells, respectively (29). In the future, it will be necessary to clarify which subpopulations among $CD14^+CD1c^+$ cells are equivalent to *bona fide* DC3s, based on heterogeneous CD163 expression utilizing single-cell RNA-seq.

In conclusion, our phenotypical, transcriptional, and functional analyses showed that $CD14^+CD1c^+$ cells were distinct DC subsets from cDC2s even in the same $CD1c^+$ population, and that the characteristics of $CD14^+CD1c^+$ cells were similar to those of recently described DC3s (29–31). Therefore, our results provide further proof for the utilization of the humanized mouse model, which enables the reconstruction of human DC heterogeneity as cDC2s and DC3s within the $CD1c^+$ population. Given the current lack of DC3 counterparts in mice and the recent reports of DC3-specific progenitor cells (31, 32), our humanized mouse model is expected to provide a useful platform to clarify *in vivo* ontogeny and dynamics of DC3s. Additionally, this humanized mouse model may also be helpful to investigate the response of DC3s to specific pathogens in future studies.

DATA AVAILABILITY STATEMENT

We have deposited RNA-seq data in Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra). Accession number is PRJNA687607.

ETHICS STATEMENT

Human umbilical cord blood samples were obtained from donors after receiving written informed consent, and they were donated by the Japanese Red Cross Society Kanto-Koshinetsu Block Blood Center (Tokyo, Japan). Human peripheral blood samples were obtained from healthy Japanese adult volunteers, after receiving written informed consent. The use of human cord blood and peripheral blood was approved by the Medical Research Ethics Committee of the NIID for the Use of Human Subjects (protocol numbers 835 and 887, respectively). All mice were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the NIID (protocol numbers 117027, 118056, and 119024).

AUTHOR CONTRIBUTIONS

RIwab, KT, and YT: study design. RIwab, KI, KT, and YT: data curation. RIwab, KT, RW, and RIwak: acquisition of data. RIwab, KI, KT, RW, YT-Y, and YT: analysis and interpretation of data. RIwab, KI, KT, RW, HM, YT-Y, HT, and YT: validation. RIwab and KI: writing the original manuscript. KT and YT: review and/or revision of the manuscript. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.643040/full#supplementary-material

Supplementary Table 1 | List of hNOJ mice used in the present study.

Supplementary Table 2 | Gene signatures used in GSEA analysis.

Supplementary Table 3 | List of differentially expressed genes in the present study.

Supplementary Figure 1 | Flow cytometric gate setting of human DC and monocyte populations in the present study. Cells were prepared from the spleen of hNOJ mice following IVT and human peripheral blood. **(A)** A representative gating strategy for $CD141^+$ population, $CD1c^+$ population, and $CD1c^-CD141^-$ (DN) population of hNOJ mice. **(B)** Representative flow cytometry profiles for cDC1s, cDC2s, $CD14^+CD1c^+$ cells, and $CD14^{high}$ monocytes with anti-CD14 monoclonal antibody (mAb) staining (upper panels) and with its isotype control staining (lower panels). **(C)** A representative flow cytometry profile for $CD14^{high}CD16^-$ classical monocytes in DN population of human PBMCs under the same staining condition of hNOJ mice samples. **(D)** Representative flow cytometry profiles for $CD14^{high}$ and $CD14^{low}$ cells in DN population of hNOJ mice. The histogram shows the PE-fluorescence intensity of $CD14^{high}$ cells (red: anti-CD88 mAb staining, orange: isotype control staining) and $CD14^{low}$ cells (blue: anti-CD88 mAb staining, green: isotype control staining).

Supplementary Figure 2 | Characterization of human DC and monocyte populations in humans and hNOJ mice. Cells were prepared from human peripheral blood and the spleen of naïve hNOJ mice or hNOJ mice following IVT. **(A)** Representative flow cytometry profiles for $CD1c^+$ population and DN population of human PBMCs. **(B)** A representative histogram profile of CD88 expression on $CD1c^+$ population and DN population of human PBMCs [red: $CD14^{low}CD1c^+$ cells, orange: $CD14^-CD1c^+$ cells (cDC2), blue: $CD14^{high}CD1c^+$ cells, $CD14^{high}CD16^-$ DN cells (classical monocyte)]. **(C)** Representative histogram profiles of CD163 expression on $CD14^{low}CD1c^+$ cells, cDC2s, and classical monocytes in human PBMCs (red: test marker staining, blue: isotype control staining). The percentages in each panel show the mean ± SD of marker positive cells in each population ($n = 5$). **(D)** Individual percentages of $CD14^+(CD14^{low})CD1c^+$ cells and cDC2s within $CD1c^+$ population in hNOJ mice ($n = 15$) and humans ($n = 5$). A significant difference ($^{***}P < 0.001$) was determined using the Mann-Whitney *U* test. **(E)** Individual percentages of $CD14^+CD1c^+$ cells, cDC2s, cDC1s, and monocytes within human $CD45^+$ cells in naïve hNOJ mice ($n = 5$) and IVT-hNOJ mice ($n = 15$). Significant differences ($^{**}P < 0.01$, $^{***}P < 0.001$) were determined using the Mann-Whitney *U* test.

Supplementary Figure 3 | Expression of IFN-γ, IL-4, and IL-17A in $CD4^+$ T cells co-cultured with human DC subsets. **(A)** Gating strategy for IFN-γ^+ cells (upper panels) and IL-4^+ cells (lower panels) using identical cDC2 co-cultured $CD4^+$ T cell samples stained with test and isotype antibodies. **(B)** Individual percentages of IFN-γ^+CTV^{low} cells (Th1; $n = 3$) and IL-4^+CTV^{low} cells (Th2; $n = 3$) within total $CD4^+$ T cells. The Same color symbols show the same donor-derived hNOJ mice. The repeated-measures one-way ANOVA followed by the Holm-Sidak's multiple comparison test was used, and no significant differences were observed. **(C)** Representative flow cytometry profiles of IL-$17A^+$ cells within $CD4^+$ T cells. **(D)** Individual percentages of IL-$17A^+CTV^{low}$ cells within total $CD4^+$ T cells and IL-$17A^+$ cells within $CTV^{low}CD4^+$ T cells. The same color symbols show the same donor-derived hNOJ mice. The repeated-measures one-way ANOVA followed by the Holm-Sidak's multiple comparison test was used, and no significant differences were observed.

Supplementary Figure 4 | Hierarchical clustering analysis among $CD1c^+$ DC subsets and monocyte-related subsets in hNOJ mice and humans. Heatmap visualization of the z-scores for the 1,000 most variable genes among hNOJ mice samples [$CD14^+CD1c^+$ cell ($n = 3$), cDC2 ($n = 3$), and monocyte ($n = 2$)] and human samples [$CD5^+$ cDC2 ($n = 4$), DC3 ($n = 4$), classical monocyte (cMo; $n = 4$), intermediate monocyte (iMo; $n = 4$), non-classical monocyte (ncMo; $n = 4$), monocyte-derived DC (MoDC; $n = 3$), monocyte-derived macrophage (MDM; $n = 3$), and Langerhans cell (LC, $n = 3$)] using the hierarchical clustering analysis.

Supplementary Figure 5 | Flow cytometric gate setting of IL-6^+ cells and TNF-α^+ cells in cell subsets. Cells were prepared from the spleen of IVT-hNOJ mice following intraperitoneal LPS injection. **(A)** Representative flow cytometry profiles for $CD14^+CD1c^+$ cells, cDC2s, cDC1s, and monocytes with anti-IL-6 mAb staining (left panels) and with its isotype control staining (right panels). **(B)** Representative flow cytometry profiles for $CD14^+CD1c^+$ cells, cDC2s, cDC1s, and monocytes with anti-TNF-α mAb staining (left panels) and with its isotype control staining (right panels).

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Conventional Type 1 Dendritic Cells (cDC1) in Human Kidney Diseases: Clinico-Pathological Correlations

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Background: cDC1 is a subset of conventional DCs, whose most recognized function is cross-presentation to $CD8^+$ T cells. We conducted this study to investigate the number and location of cDC1s in various human kidney diseases as well as their correlation with clinico-pathological features and $CD8^+$ T cells.

Methods: We analyzed 135 kidney biopsies samples. Kidney diseases included: acute tubular necrosis (ATN), acute interstitial nephritis (AIN), proliferative glomerulonephritis (GN) (IgA nephropathy, lupus nephritis, pauci-immune GN, anti-GBM disease), non-proliferative GN (minimal change disease, membranous nephropathy) and diabetic nephropathy. Indirect immunofluorescence staining was used to quantify cDC1s, $CD1c^+$ DCs, and $CD8^+$ T cells.

Results: cDC1s were rarely present in normal kidneys. Their number increased significantly in ATN and proliferative GN, proportionally much more than $CD1c^+$ DCs. cDC1s were mainly found in the interstitium, except in lupus nephritis, pauci-immune GN and anti-GBM disease, where they were prominent in glomeruli and peri-glomerular regions. The number of cDC1s correlated with disease severity in ATN, number of crescents in pauci-immune GN, interstitial fibrosis in IgA nephropathy and lupus nephritis, as well as prognosis in IgA nephropathy. The number of $CD8^+$ T cells also increased significantly in these conditions and cDC1 number correlated with $CD8^+$ T cell number in lupus nephritis and pauci-immune GN, with many of them closely co-localized.

Conclusions: cDC1 number correlated with various clinic-pathological features and prognosis reflecting a possible role in these conditions. Their association with $CD8^+$ T cells suggests a combined mechanism in keeping with the results in animal models.

Keywords: dendritic cells, $CD141^+$ DCs, conventional DCs, crescent, interstitial fibrosis, glomerulonephritis, acute tubular necrosis

INTRODUCTION

Dendritic cells (DCs) are the central orchestrators of effective immunity. These cells are heterogeneous and can be divided into distinct subsets based on their phenotype and function. Dendritic cells can be broadly categorized into plasmacytoid DCs (pDC) and conventional DCs (cDC). cDC consists of two major subsets: cDC1 ($CD141^+$ DCs in humans and $CD103^+$ or $CD8\alpha^+$ DCs in rodents) and cDC2 ($CD1c^+$ DCs in human and $CD11b^+$ DCs in rodents) (1). cDC1 were discovered first in mouse and then in humans and are characterized by their superior ability to phagocytose necrotic cells through damage-associated molecular pattern (DAMP) receptor Clec9A and to cross-present to $CD8^+$ T cells. In contrast, cDC2s are effective $CD4^+$ T cell activators but inferior at $CD8^+$ T cell activation (2).

DCs have been studied in human kidney disease and their number was found to be increased in glomerulonephritis (GN) (3, 4). After the discovery of cDC1, accumulating animal studies have shown that they play a pivotal role in kidney diseases, such as in adriamycin nephropathy and crescentic GN, through interaction with T cells (5–8). However, such studies are lacking in human kidney disease, with only one study demonstrating increased numbers of cDCs in GN (9). We conducted this study to provide analysis of the key cDC subsets, cDC1 and cDC2, in a wide range of human kidney diseases including non-glomerular diseases [acute tubular necrosis (ATN), acute interstitial nephritis (AIN)], proliferative GN (IgA nephropathy, lupus nephritis, pauci-immune GN, anti-GBM disease), non-proliferative GN (minimal change disease (MCD), membranous nephropathy) and diabetic nephropathy. We found cDC1s to be significantly correlated with pathological features including severity of ATN, crescent formation in pauci-immune GN and interstitial fibrosis in immune-mediated GN. In addition, consistent with their specialized ability to activate $CD8^+$ T cells in animal models, we also demonstrated their correlation with $CD8^+$ T cells in these samples. These findings provide an impetus to explore new therapeutic targets that manipulate these cells for treatment of kidney diseases.

MATERIAL AND METHODS

Patients and Tissue Samples

The study was conducted in accordance with principles of the Declaration of Helsinki and was approved by the Human Research Ethics Committee of the Western Sydney Local Health District. Informed patient consent was obtained.

We analyzed 176 frozen diagnostic kidney biopsy samples taken from non-pregnant adult patients (>18 years old) between 1st June 2016 to 30th June 2017 in Westmead Hospital, Sydney, Australia. Kidney tissues were snap frozen in O.C.T. compound (Tissue-Tek Sakura, USA) and stored at -80°C. Forty-one (41) samples had poor tissue quality and were excluded from the study. Baseline patient data including age, gender, estimated glomerular filtration rate (eGFR) and degree of proteinuria were collected at the time of kidney biopsy. eGFR was calculated using the CKD-EPI formula. The diagnosis was made by a renal pathologist, based on light, immunofluorescence (IF) and electron microscopy as well as clinical history.

A variety of diseases were analyzed including non-glomerular diseases (ATN, AIN), proliferative GN (IgA nephropathy, lupus nephritis, pauci-immune GN, anti-GBM disease), non-proliferative GN (MCD, membranous nephropathy) and diabetic nephropathy. For ATN, we only included non-septic cases as septic ATN has a different pathophysiology. ATN was further categorized into mild-moderate disease (defined as <50% cortical tubules showing injury) and severe disease (>=50% cortical tubules showing injury). IgA nephropathy was classified according to the Oxford MEST score (M mesangial hypercellularity, E endocapillary hypercellularity, S segmental glomerulosclerosis, T tubular atrophy/interstitial fibrosis). We also analyzed the correlation between cDC1 number and prognosis (defined *a priori* as >20% reduction in eGFR on or before 31st December 2019) in IgA nephropathy by dividing patients into 2 groups according to cDC1 number with cut-off point at upper quartile (>=15). Lupus nephritis was further classified into 2 groups according to the level of interstitial fibrosis (<25%, >=25% cortical interstitial involvement). Pauci-immune GN was classified into 2 groups according to the percentage of glomeruli with crescents (<40%, >=40%).

As normal kidney controls, we used 5 normal renal cortices from nephrectomy samples as well as 2 donor kidneys not suitable for transplant. For tumor nephrectomies, samples were taken from the pole opposite to the tumor and at least 5 cm from the tumor margin. These tissues had normal macroscopic appearance. Microscopically, none of these kidney samples had evidence of significant glomerular or tubulointerstitial inflammation or injury. We used normal adult donor splenic tissue as positive control for testing antibodies.

Immunofluorescence Staining

Serial cryostat sections were cut at 5μm and placed onto Superfrost Ultra Plus glass slides (Thermo Scientific, USA). Slides were stored at -80°C. Tissue sections were fixed with 100% methanol at -20°C for 10 minutes and then air-dried. Indirect immunofluorescence staining was performed using the following method: tissue sections were washed in DPBS (Lonza, USA) and blocked with 2% Bovine Serum Albumin (Sigma-Aldrich, USA) for 15 minutes; then they were stained with primary antibody at 4°C overnight followed by secondary antibodies for 40 minutes at room temperature. **Table 1** is a list of primary and secondary antibodies used and their dilutions. Nuclei were stained with DAPI (1:250,000, ThermoFisher, USA) before samples were mounted on coverslips with fluorescence mounting medium (Dako, USA). Non-specific staining and cross reactivity between different primary and secondary antibodies were checked and excluded.

cDC1s were identified by staining for marker Clec9A. In humans, Clec9A expression is highly restricted to cDC1s in blood and tissues (10, 11). To confirm this in the kidneys, we performed double staining of Clec9A and HLA-DRB1 as well as Clec9A and CD11c in selected normal and diseased conditions. Most, if not all Clec9A overlapped with HLA-DRB1 (**Supplementary Figure 1**) and CD11c (**Supplementary Figure 2**),

TABLE 1 | Primary and secondary antibodies used in the study.

Primary antibody	Host	Clone	Company	Dilution
Clec9A	Mouse anti-Human	8F9	Miltenyi Biotec	1:25
CD1c	Mouse anti-Human	L161	Biolegend	1:100
HLA-DRB1	Rabbit anti-Human	EPR1126	Abcam	1:100
CD11c	Rabbit anti-human	EP1347Y	Abcam	1:100
CD8	Rabbit anti-Human	SP16	Invitrogen	1:100
Secondary antibody				
Alexa Fluor 647	Donkey anti-Rabbit		Invitrogen	1:1,600
Alexa Fluor 546	Goat anti-Mouse		Invitrogen	1:1,600
Alexa Fluor 546	Goat anti-Rabbit		Invitrogen	1:1,600

indicating that Clec9A accurately identified the cDC1 subset. cDC2 were identified by staining for marker CD1c and double staining of CD1c with HLA-DRB1 and CD11c was also performed to ensure accuracy. $CD8^+$ T cells were identified by staining for marker CD8.

Quantification of DC Numbers and Statistical Analysis

IF images were acquired with a confocal microscope (Olympus FV 1000 confocal laser scanning microscope). Four randomly selected pictures were taken for each sample under 20X magnification and the average number of positive cells in these 4 pictures was used in the analysis. Labeled cells were counted by an investigator twice in a blinded fashion and the mean value was used. When calculating cDC2/cDC1 ratio, we used the mean number of cDC2 and cDC1 for each sample. When cDC1 number was 0, we used 1 instead of 0 in calculating cDC1/cDC1 ratio as it would otherwise be infinite. The number of cells was expressed per high power field. In quantifying the number of cells in the intra-glomerular region, 4 randomly selected glomeruli were taken from each sample and the average number of cells inside these 4 glomeruli was calculated. Image J manual cell counting and marking tool was used to record positive cells.

Statistical analysis was performed using SPSS version 26. All analyses were 2 tailed and $P<0.05$ was considered statistically significant. When there was multiple comparisons to the control group (5), Bonferroni adjustment was made and $P<0.01$ was considered statistically significant. Continuous variables were presented as median with interquartile range (IQR). The distributions between groups were compared using Mann-Whitney U-test or Kruskal Wallis tests as appropriate. The strength of association was quantified using Spearman rank correlation. Kaplan-Meier time-to-event curves with log-rank test were used for outcome analysis.

RESULTS

Patient's Baseline Characteristics

The baseline characteristics of patients in control and disease cohorts are summarized in **Table 2**. A total of 135 patients were included in the study. A wide range of kidney diseases were analyzed including non-glomerular diseases [ATN (22), AIN (10)], proliferative GN [IgA nephropathy (44), lupus nephritis (12), pauci-immune GN (12), anti-GBM disease (4)], non-proliferative GN [MCD (5), membranous nephropathy (5)] and diabetic nephropathy (21). There were more females in the lupus nephritis group and their age tended to be younger compared with other kidney diseases, which is consistent with literature (12). Patients with pauci-immune GN and anti-GBM disease had the lowest eGFR. The proteinuria level was the highest in MCD and membranous nephropathy.

Number and Location of DCs in Control and Disease

cDC1s were rarely present in normal kidneys (**Figure 1**) and cDC2 numbers were approximately 7 times the number of cDC1.

The number of cDC1 increased significantly in ATN and proliferative GN (**Figure 2A**), while their number remained unchanged compared to control in AIN, membranous

TABLE 2 | Baseline characteristics.

	Patient number	Gender (Male)	Age (years)	eGFR (ml/min/1.73^2)	Proteinuria (g/24 hours)
Control	7	4	54 (40-64)	86 (79-90)	N/A
Non glomerular disease					
ATN	22	16	59 (43-69)	22 (18-40)	0.6 (0.2-0.8)
AIN	10	7	48 (26-72)	28 (21-36)	0.4 (0.3-0.7)
Proliferative GN					
IgA	44	31	47 (37-63)	43 (31-73)	2.2 (0.9-4.0)
Lupus nephritis	12	4	29 (26-37)	71 (30-90)	3.2 (0.8-5.1)
Pauci-immune	12	9	67 (61-77)	14 (8-30)	4.1 (1.4-5.0)
Anti-GBM	4	3	57 (33-66)	13 (4-23)	N/A
Non Proliferative GN					
Minimal change disease	5	1	50 (48-62)	82 (63-90)	10.2 (5.0-14.5)
Membranous nephropathy	5	2	51 (38-78)	33 (14-77)	4.3 (2.9-10.8)
Other non immune mediated glomerular disease					
Diabetic nephropathy	21	11	68 (47-74)	33 (23-55)	3.3 (2.0-6.5)

Data are expressed as median (IQR). N/A indicates data not available.

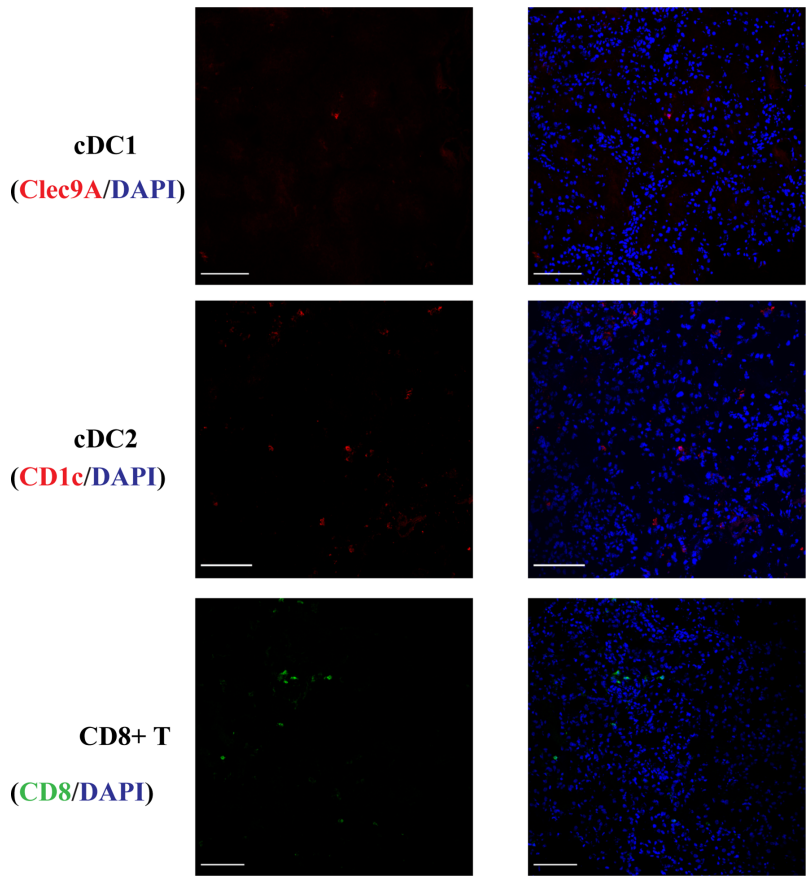

FIGURE 1 | Normal kidney cDC1, cDC2 and $CD8^+$ T cells. DCs were rarely present in normal kidneys and cDC2 numbers were approximately 7 times the number of cDC1. (Bar = 100 μm).

nephropathy, MCD and diabetic nephropathy (**Supplementary Figure 3**). The number of cDC2 also increased significantly in ATN and proliferative GN (**Figure 2B**). There was a reduction in the cDC2/cDC1 ratio indicating cDC1 increased proportionally more than cDC2 (**Figure 2C**).

Most cDC1s were located in the interstitium, except in lupus nephritis, pauci-immune GN and anti-GBM disease where they were also found in peri-glomerular and intra-glomerular regions (**Figure 3A**). In addition, we also found a significant number of $CD8^+$ T cells in peri-glomerular and intra-glomerular regions (**Figure 3A**), and many of them co-localized with cDC1s (**Figure 3B**). On the other hand, cDC2s were rarely found in intra-glomerular regions and there was minimal co-localization with $CD8^+$ T cells.

Association Between cDC1 With Clinical-Pathological Features and $CD8^+$ T Cells

We analyzed the correlation between cDC1 and clinicopathological features as well as $CD8^+$ T cells.

There were 22 cases of ATN (mild - mod disease n=12, severe disease n=10). More severe disease was associated with a higher number of cDC1 (p=0.032), but not cDC2 (**Figure 4A**). cDC1 increased proportionally more than cDC2 (cDC2/cDC1 ratio 2.5, p=0.019). The number of $CD8^+$ T cells also increased significantly in ATN (p=0.005) (**Table 3**, **Figure 2D**). The number of cDC1 did not correlate with $CD8^+$ T cell number in ATN.

Forty-four cases of IgA nephropathy were analyzed. Using the Oxford classification MEST score (M mesangial hypercellularity, E endocapillary hypercellularity, S segmental glomerulosclerosis, T tubular atrophy/interstitial fibrosis), a higher number of cDC1 was associated with a higher T score (p=0.008), but not MES scores (**Figure 4B**). There were 7 cases with crescents and the number of cCD1 was not associated with the number of crescents. The number of kidney $CD8^+$ T cells was significantly higher in IgA nephropathy than control (p<0.001) (**Table 3**, **Figure 2D**). There was no correlation between cDC1 and $CD8^+$ T cell number. Thirty-five (35) patients had follow up data on or before 31st December 2019, of whom 9 experienced > 20% reduction in eGFR. Dividing patients into 2 groups according to cDC1 number with cut-off point at upper quartile (>=15), the higher cDC1 number group was associated with worse outcome (**Figure 4E**).

There were 12 cases of lupus nephritis. As in IgA nephropathy, higher cDC1 number was associated with more severe fibrosis (p=0.020) (**Figure 4C**). In addition, $CD8^+$ T cell

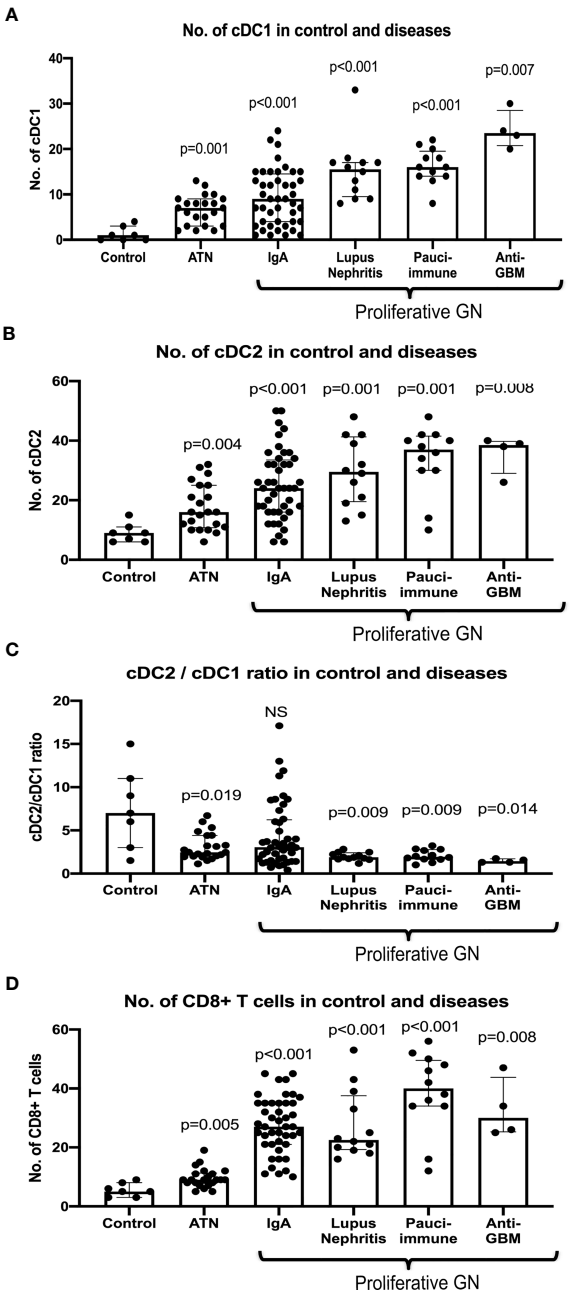

FIGURE 2 | Number of cDC1 and cDC2, cDC2/cDC1 ratio and $CD8^+$ T cell in control and selected diseases. P value is calculated for each disease *versus* control. Both cDC1 and cDC2 increased significantly in ATN, IgA, lupus nephritis, pauci-immune GN and anti-GBM disease **(A, B)** with cDC1 increased proportionally more than cDC2 in ATN, lupus nephritis, pauci-immune GN and anti-GBM disease **(C)**. $CD8^+$ T cells were increased significantly in ATN, IgA, lupus nephritis, pauci-immune GN and anti-GBM disease **(D)**.

number also increased significantly (P<0.001) (**Table 3**, **Figure 2D**) and this correlated with the number of cDC1 cells (r=0.614, p=0.034) (**Table 3**). A significant number of cDC1 and $CD8^+$ T cells were found in peri-glomerular as well as intra-glomerular regions (**Figure 3A**). Many cDC1s co-localized with $CD8^+$ T cells (**Figure 3B**).

Twelve cases of pauci-immune GN were included in the analysis. A higher number of cDC1 was found in the group with crescents >40% (p=0.021*)* (**Figure 4D**). The number of $CD8^+$ T cells was also significantly higher than in control kidneys (p<0.001) (**Table 3**, **Figure 2D**). The number of cDC1s correlated with that of $CD8^+$ T cells (r=0.644, p=0.024) (**Table 3**). As in lupus nephritis, there was a prominent peri-glomerular cDC1 and $CD8^+$ T cell infiltration (**Figure 3A**). There were also intra-glomerular cDC1s and $CD8^+$ T cells (**Figure 3A**). Numerous cDC1 co-localized with $CD8^+$ T cells (**Figure 3B**).

There were 4 cases of anti-GBM disease. cDC1, cDC2 and $CD8^+$ T cell numbers increased significantly compared to control, with cDC1s increased proportionally much more than cDC2s (**Figures 2, 2D**). However, there was no correlation between cDC1 and $CD8^+$ T cell number. There were also numerous cDC1 and $CD8^+$ T cells in the peri-glomerular region as well as inside the glomerulus (**Figures 3A, B**).

DISCUSSION

This is the first study providing a detailed analysis of different cDC subsets in healthy and diseased human kidney. This study provides several important findings; first, in non-septic ATN where innate immunity plays a central role, we found higher numbers of cDC1s, which increased proportionally more than cDC2s. cDC1 number also correlated with disease severity indicating they may play a role in this condition, Second, in interstitial fibrosis associated immune-mediated disease (IgA nephropathy and lupus nephritis), we found that cDC1 number correlated with severity of fibrosis, as well as prognosis in IgA nephropathy, while no such correlation was found in non-immune mediated fibrotic disease (diabetic nephropathy). Third, there was a strong correlation between cDC1 number and crescent formation in pauci-immune GN, and cDC1 were present in large numbers in peri-glomerular and intra-glomerular regions indicating their possible role in crescent formation. Fourth, the number of cDC1 correlated with $CD8^+$ T cell numbers in lupus nephritis and pauci-immune GN, with numerous cDC1s co-localized with $CD8^+$ T cells suggesting their possible interaction. This is keeping with our findings in animal models of kidney disease that show murine homologs of cDC1 cells preferentially activate $CD8^+$ T cells. Taken together, these findings suggest that cDC1 play an important role across a range of kidney diseases including ATN, interstitial fibrosis in immune-mediated disease and crescent formation, and that they may potentially act through activation of $CD8^+$ T cells.

cDC1 Number Correlated With Disease Severity in ATN

In non-septic ATN, for the first time, we showed a significantly increased number of DCs, especially cDC1s compared with cDC2s. Importantly, the cDC1 number correlated with disease severity. In addition, $CD8^+$ T cell number was also increased.

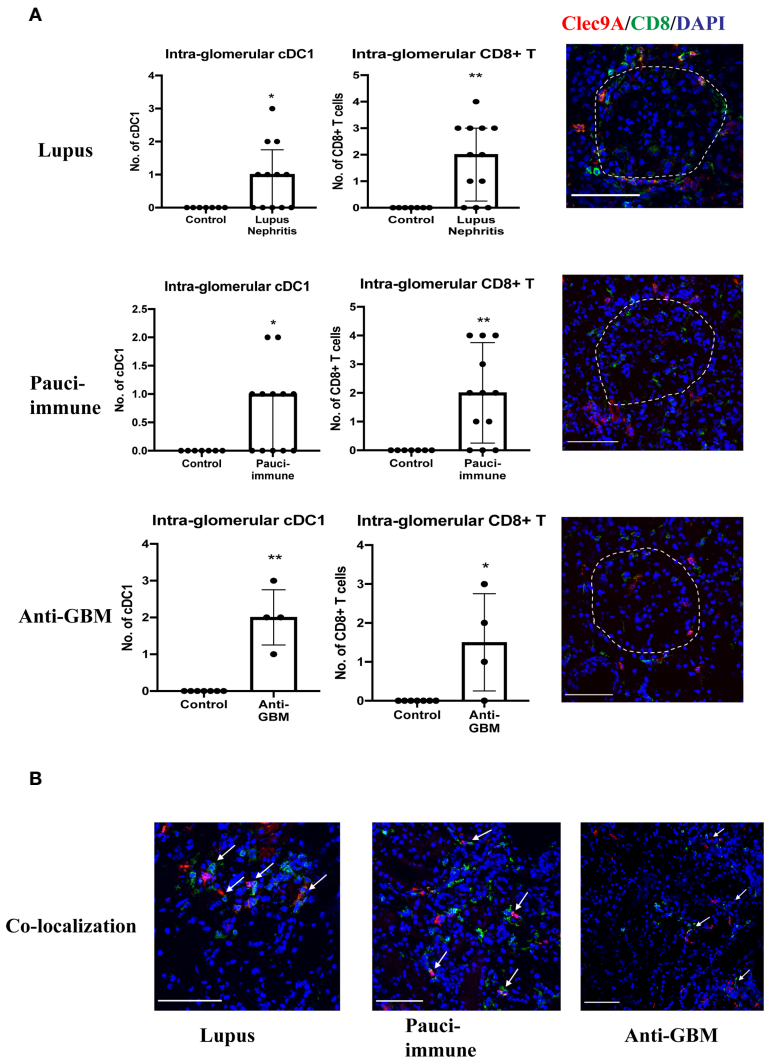

FIGURE 3 | **(A)** cDC1 and CD8$^+$ T cells in intra-glomerular regions. **(B)** cDC1 co-localization with CD8$^+$ T cells. (Bar = 100 μm). * $P < 0.05$, ** $P < 0.01$.

ATN is usually caused by ischemia reperfusion injury (IRI), nephrotoxins or sepsis. Traditionally, IRI and toxins cause sterile inflammation, and innate immunity, where DCs and T cells are less important, was considered to play a dominant role. However, increasing evidence from animal studies has shown both cDC1 and CD8$^+$ T cells are important players. Previous studies in animal IRI and cisplatin nephrotoxicity found the total DC and T cell numbers increased (13–17). Our previous studies showed in Adriamycin nephropathy, cDC1 numbers increased significantly (5). In IRI, a subset of activated dendritic cells demonstrate increased capacity to cross present antigen to CD8$^+$ T cells (18). In Adriamycin nephropathy, we also found cDC1s elicited a CD8$^+$ T cell response leading to injury (5). The lack of a correlation between cDC1 and CD8$^+$ T cell in human ATN in contrast to the findings in animal models may reflect different non-immunological pathways of injury in human ATN compared to the IRI in mouse models where cDC1s play a significant role through CD8$^+$ T cells. In addition, it has been demonstrated in rodents that cDC1 are recruited into the tissue by chemoattractant XCL1 produced by natural killer cells (19). Therefore, it may be worthwhile to study this further in kidney disease.

cDC1 Number Correlated With Immune-Mediated Interstitial Fibrosis

The second significant finding in this study is that cDC1 number correlated with the severity of interstitial fibrosis associated with immune-mediated disease (IgA nephropathy and lupus nephritis), but not in non-immune mediated fibrotic disease (diabetic nephropathy), Previous study showed increased numbers of cDC1s and cDC2s in interstitial fibrosis (9). We, for the first time, showed that this is only true in immune-mediated disease and cDC1 number increased proportionally more than cDC2s. In addition, we also demonstrated cDC1

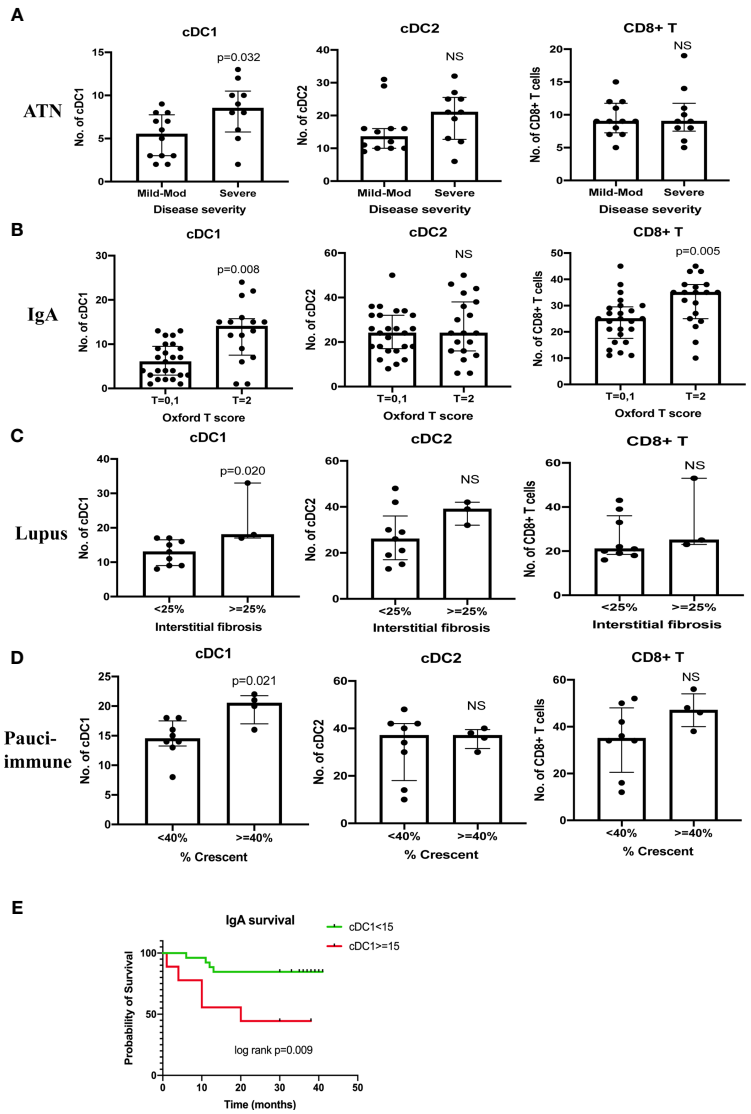

FIGURE 4 | Correlation between cDC1, cDC2 and CD8$^+$ T cell number and disease severity **(A–D)**. Kaplan Meier curve of IgA nephropathy survival **(E)**. T score 0 refers to the percentage of area showing tubular atrophy/interstitial fibrosis < = 25%, T score 1 refers to the percentage of area showing tubular atrophy/interstitial fibrosis 26 - 50%, T score 2 refers to the percentage of area showing tubular atrophy/interstitial fibrosis > 50%. Survival was defined as > 20% reduction in eGFR on or before 31st December 2019. NS, not significant.

TABLE 3 | CD8+ T cell number and correlation coefficient between cDC1 and CD8+T cells numbers in control and diseased kidneys.

	CD8$^+$ T cell number		cDC1 and CD8$^+$ T Correlation coefficient
	Median (IQR)	p	
Control	5 (3-8)	Reference	NS
ATN	9 (7-11)	0.005	NS
IgA	25 (21-34)	<0.001	NS
Lupus nephritis	22 (19-37)	<0.001	0.614 (p=0.034)
Pauci-immune	40 (34-49)	<0.001	0.644 (p=0.024)
Anti-GBM	30 (25-43)	0.008	NS

Data are expressed as median (IQR). NS indicates not significant. Statistical significance was assessed between control and disease.

number correlated with prognosis and the number of CD8$^+$ T cells also increased significantly in IgA nephropathy. This is supported by animal studies showing DCs directly contribute to fibrosis. For example, DC-derived amphiregulin promoted fibrosis (20). It is also possible that cDC1s contributed to fibrosis through CD8$^+$ T cells, which are known to contribute to fibrosis in other organs (21–23). Since interstitial fibrosis is linked to the progression of chronic kidney disease, it is not surprising that we found cDC1 number to be a good prognostic marker in IgA nephropathy. Other studies have shown CD8$^+$ T cells correlated with prognosis of IgA nephropathy (24), which may be the result of cross presentation from cDC1s. In lupus nephritis, we demonstrated a correlation between cDC1 number and

interstitial fibrosis as well as number of $CD8^+$ T cells. Previous studies have also shown an increased kidney cDC1 number in lupus nephritis (25), specially class III and VI lupus nephritis, with a corresponding reduction in their circulating numbers (26). We extended these findings by showing that they also correlated with chronic changes. It is well established that interstitial inflammation, which is comprised of T cells, B cells, dendritic cells and macrophages, has a dominant role in the progression of lupus nephritis (27). cDC1s may contribute to the progression of lupus nephritis in a variety of ways. First, activation of interferon plays a key role in the pathogenesis of lupus nephritis (28–30) and cDC1s are a prominent producer of IFN-λ. (31) Second, cDC1 may contribute to lupus nephritis progression through $CD8^+$ T cells (32–37) and our finding of a correlation between cDC1s and $CD8^+$ T further supports their possible interaction. The role of $CD8^+$ T cells in lupus nephritis has been previously demonstrated. $CD8^+$ T cells control autoreactive immunity by release of cytotoxic molecules. $CD8^+$ T cells in lupus nephritis were found to have dampened cytotoxic function, which can trigger autoimmunity (38). In addition, these cells can also generate lupus autoantigens (39). There has been abundant evidence that $CD8^+$ T cells in both kidney (32, 33) and urine (34–36) correlate with disease activity and histological injury in lupus nephritis. In addition, $CD8^+$ T-cell exhaustion was shown to predict a favorable prognosis (37).

cDC1 Number Correlated With the Number of Crescents in Pauci-Immune GN

In pauci-immune GN, we found cDC1s aggregated in the peri-glomerular and intra-glomerular regions and their number correlated with the number of crescents and $CD8^+$ T cells. Previous studies showed that DCs are rarely present inside the glomerulus (3, 4, 9) or only in very small numbers (40). On the other hand, T cells were prominent in interstitium, peri-glomerular and intra-glomerular regions (41–44). We found that both cDC1s and $CD8^+$ T cells are prominent in the peri-glomerular and intra-glomerular regions with many of them co-localized. In addition, cDC1 number correlated with crescent and $CD8^+$ T cell number. All of these findings suggest a role for cDC1 in crescent formation through interaction with $CD8^+$ T cells. The pathogenic role of $CD8^+$ T cells in pauci-immune GN and crescent formation has already been demonstrated in animal models (45, 46). Consistent with our findings in humans, in animal crescentic GN, cDC1 and $CD8^+$ T cells were found especially in the periglomerular region (47, 48). It has been shown that Bowman's capsule provides a protected immunological niche by preventing access of DCs and cytotoxic $CD8^+$ T cells to Bowman's space and thereby podocytes (45, 47). However, when Bowman's capsule was breached, these inflammatory cells gained access and destroyed podocytes resulting in rapidly progressive GN (47).

One limitation of this study is that the IF staining technique allows the use of only a limited number of markers. It would be beneficial to extend our findings using technology such as flow cytometry, which can combine multiple markers to further analyze the phenotype of these DCs and their relevant cytokine and chemokine profiles. However, location information will be lost. Other techniques such as multiplex immunohistochemistry and Nanostring can also be considered in future studies to further examine these cells in kidney disease. In addition, when staining cDC2s using CD1c, HLA-DRB1 and CD11c, there may be a small percentage of B cells that express these markers as well, which has not been ruled out.

CONCLUSIONS

Even though cDC1 comprise a minor subset of DCs under homeostatic conditions, this study demonstrates significant correlation between this cell population and clinico-pathological features in human kidney disease. This reflects their likely importance in disease processes such as ATN, crescent formation in proliferative GN and interstitial fibrosis in immune-mediated GN. In addition, their co-localization and correlation with $CD8^+$ T cells may provide an explanation for their mechanism of action, corroborating data from animal models. These findings provide an impetus to explore new therapeutic targets that manipulate these cells for treatment of kidney diseases, as we have done in animal studies (6), and to investigate their use as a prognostic marker. Further studies in both humans and animals are needed to interrogate the role of cDC1s, their mechanism of action and how best to target them therapeutically.

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

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of the Western Sydney Local Health District. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YPW, QC, TC, and DH designed the study. QC, TC, RW, JW, and FA carried out experiments; QC, HY, CP, and TC interpreted and analyzed the data. TC made the figures. NR,

DH, YMW, VL, SA, and GZ provided intellectual input and expertise; MP collected nephrectomy tissue samples. TC, QC, YPW, and DH drafted and revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.635212/full#supplementary-material

Supplementary Figure 1 | Double staining of Clec9A with HLA-DRB1.

Supplementary Figure 2 | Double staining of Clec9A with CD11c.

Supplementary Figure 3 | Number of cDC1 in AIN, membranous, MCD and diabetic nephropathy, which didn't show an increase in these conditions.

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REVIEW
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Recent Advances and Future Perspective of DC-Based Therapy in NSCLC

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Current treatment for patients with non-small-cell lung cancer (NSCLC) is suboptimal since therapy is only effective in a minority of patients and does not always induce a long-lasting response. This highlights the importance of exploring new treatment options. The clinical success of immunotherapy relies on the ability of the immune system to mount an adequate anti-tumor response. The activation of cytotoxic T cells, the effector immune cells responsible for tumor cell killing, is of paramount importance for the immunotherapy success. These cytotoxic T cells are primarily instructed by dendritic cells (DCs). DCs are the most potent antigen-presenting cells (APCs) and are capable of orchestrating a strong anti-cancer immune response. DC function is often suppressed in NSCLC. Therefore, resurrection of DC function is an interesting approach to enhance anti-cancer immune response. Recent data from DC-based treatment studies has given rise to the impression that DC-based treatment cannot induce clinical benefit in NSCLC by itself. However, these are all early-phase studies that were mainly designed to study safety and were not powered to study clinical benefit. The fact that these studies do show that DC-based therapies were well-tolerated and could induce the desired immune responses, indicates that DC-based therapy is still a promising option. Especially combination with other treatment modalities might enhance immunological response and clinical outcome. In this review, we will identify the possibilities from current DC-based treatment trials that could open up new venues to improve future treatment.

Keywords: dendritic cells, lung cancer, immunotherapy, immunology and lung cancer, non-small cell lung cancer

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide (1). This type of cancer is a heterogeneous disease (2). Based on histology, lung cancer is divided into small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC is the most prevalent form, accounting for about 80-85% of the lung cancer cases (3). The five-year overall survival rate (OS) for NSCLC is around 20% in the western world, highlighting the importance to explore the current and future therapeutic approaches in this field (4, 5).

Although surgery remains the cornerstone of therapy for early-stage NSCLC, a wide range of therapeutic options for adjuvant treatment or treatment of advanced stage disease have been introduced over the last decade. Targeted therapy and immunotherapy are examples of these novel therapies (6, 7). Targeted therapy targets specific alterations in NSCLC cells that stimulate tumor growth, for example mutations in the epidermal growth factor receptor (EGFR). Many other specific targets in NSCLC have been identified over time. Targeted therapy often leads to prolonged survival and greatly enhanced quality of life in this subgroup of patients (8, 9). Most patients with NSCLC lack actionable therapy targets. Therefore, immunotherapy, with or without chemotherapy, is the first-line treatment for the majority of NSCLC patients with advanced stage disease. Although targeted therapy and immunotherapy greatly improved clinical outcome in NSCLC, not all patients respond (10). Moreover, the patients who do respond eventually develop therapy resistance. Therefore, a high clinical need for new systemic treatment modalities remains. During this review we will shine our light on the rather unexposed field of dendritic cell (DC)-based therapies, to explore whether these could be a valuable treatment option for NSCLC.

IMMUNOTHERAPY IN NSCLC

The role of the immune system in prevention of cancer development and progression has been widely recognized. Immunotherapy exploits this role by stimulating the patient's immune system to eliminate the tumor. Different immunotherapeutic strategies are being used or currently studied for their use in cancer. These can be largely subdivided into cancer vaccines, cellular therapies, immune stimulatory agonists and immune checkpoint inhibitors (ICIs). NSCLC is a promising potential target for immunotherapeutic approaches due to its high tumor mutational burden, which enhances immunogenicity of the tumor (11). ICIs are the only currently approved immunotherapy option for NSCLC. The most frequently used ICIs are directed against programmed-death receptor 1 (PD-1), expressed on immune effector cells such as T cells and natural killer (NK) cells, or its ligand programmed-death ligand 1 (PD-L1), which is expressed on antigen-presenting cells (APCs) and tumor cells. Receptor binding of PD-1 can lead to inhibition of effector cell function and survival, while it induces T regulatory cells (Tregs) (12–14). Immune cells in tumors frequently demonstrate a non-functional or 'exhausted' phenotype which hampers an anti-cancer immune response. ICIs aim to revert this immunosuppressive phenotype, thereby inducing an efficient anti-cancer immune response (15).

Recently, anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4), which is another ICI, has been registered to be used in combination with a PD-1 inhibitor in the United States and is registered for the combination with a PD-1 inhibitor and chemotherapy in Europe (16, 17). CTLA-4 is expressed on T cells after activation. It is also constitutively expressed on Tregs. CTLA-4 binds CD80 and CD86 on APCs. Receptor binding transmits an inhibitory signal to the T cell. Furthermore, binding of CTLA-4 to CD80 and CD86 blocks their binding to T cell receptors thereby hampering T cell activation (18).

Nowadays, it is known that the tumor and its surrounding microenvironment (TME) can modulate anti-tumor immune responses. Recent data suggest that the low response rate to ICIs could be partly explained by the lack of immune cells in the TME or other regulatory factors that prevent an anti-tumor immune response (19). Therefore, other forms of immunotherapy to enhance the anti-tumor immune response are currently being studied, such as DC-based therapy.

DC-BASED THERAPY

DC-based therapy depends on the fundamental link that DCs form between tumor antigen recognition and an anti-tumor immune response. More specifically, DCs are highly specialized APCs that show the highest antigen-presenting potential when inducing naïve T cell activation (20). In tissue, DCs are constantly scanning their surroundings. Upon antigen encounter in the presence of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMP), DCs get activated, undergo maturation and secrete large amounts of pro-inflammatory cytokines to shape the local inflammatory environment (6, 21, 22). After maturation, DCs migrate to the lymph node where they activate T cells to induce an immune response directed against their presented antigen. In absence of PAMP or DAMP signals during antigen encounter, DCs remain immature, migrate to the lymph nodes and induce antigen-specific tolerance in T cells. DC-based therapies showed promising results in several malignancies such as melanoma, prostate cancer, and glioma (23–25). In NSCLC only early-phase clinical trials have been performed, which show disappointing clinical results but were not powered to evaluate clinical effect. In this review, we will analyse these studies and discuss different possibilities to optimize DC-based therapy in order to improve therapeutic effects.

DC VACCINATION MONOTHERAPY FOR NSCLC

The DC-based therapy in NSCLC consists of the vaccination of patients with DCs. In all studies investigating DC vaccination in NSCLC patients, monocytes derived from autologous peripheral blood mononuclear cells (PBMCs) were differentiated to moDCs *ex vivo* (**Table 1**) (26–36). These DCs were then primed with a combination of several synthetic peptides of commonly expressed tumor antigens in NSCLC or autologous tumor lysate. In the majority of studies, primed DCs were administered *via* multiple subcutaneous injections. *In vivo*, these DCs are supposed to activate cytotoxic T cells that will induce a tumor-directed immune reaction.

All studies showed DC vaccination to be safe. In addition, many studies examined vaccine-specific immunological responses by determining *ex vivo* T cell responses directed

TABLE 1 | Characteristics of studies in which DC vaccination monotherapy was performed in NSCLC.

Ref.	Subject number	Clinical stage	Antigen source	DC maturation status	Type and regimen of DC administration	Most important results after vaccination
Ueda et al., Int. J. Oncol. (26)	N = 3	III and IV[a]	CEA peptide	Immature	5 biweekly i.d. and s.c. vaccinations	• 2 out of 3 patients showed a DTH response.
Hirschowitz et al., J. Clin. Oncol. (27)	N = 16	I, II and III	Irradicated tumor lysate or lysate of a NSCLC cell line	Mature	2 i.d. vaccinations	• 6 out of 16 patients showed tumor-specific IFN-γ T cell responses. • No correlation between immunological response and OS or DFS was determined.
Chang et al., Cancer (28)	N = 6	III and IV	Tumor lysate	Not fully mature[b]	4 weekly vaccinations followed by 2 biweekly boost vaccinations in the inguinal lymph nodes	• 2 out of 6 patients demonstrated increased tumor-specific IFN-γ T cell responses. • These 2 patients demonstrated stable disease.
Hirschowitz et al., Lung cancer (29)	N = 14	I, II and III	Irradicated tumor lysate or lysate of a NSCLC cell line	Immature	2 i.d. vaccinations	• 10 out of 14 patients showed tumor-specific IFN-γ T cell responses. • No correlation between immunological response and OS or DFS was demonstrated.
Um et al., Lung cancer (30)	N = 9	IIIB and IV[a]	Tumor lysate	Mature	3 i.d. DC vaccinations at 2 weeks interval	• 5 out of 9 patients showed increased tumor-specific IFN-γ T cell responses. • All patients demonstrated disease progression.
Perroud et al., J. Exp. Clin. Cancer Res. (31)	N = 5	III and IV	Peptides of WT-1, MAGE-1, and Her-2/neu	Unknown[c]	2 biweekly s.c. and i.v. vaccinations	• *Ex vivo*, T cell responses directed towards the DC vaccine were increased. • 2 out of 5 patients showed an unexpectedly long OS.
Engell-Noerregaard et al., World J. Of Vaccine (32)	N = 22	III and IV	Lysate of a melanoma cell line expressing among others MAGE-A/B	Mature	A weekly s.c. vaccination for 5 weeks, followed by a booster vaccination after 6 weeks, with s.c. IL-2, COX-2 inhibitors, and TLR7 agonist[d]	• Ex vivo, vaccination-specific IFN-γ T cell responses were mostly observed in patients showing stable disease. • Some patients showed unexpected long survival.
Takahashi et al., Eur. J. Cancer (33)	N = 47	II, III and IV[e]	Tumor lysate or multiple peptides of WT-1, MUC1, and CEA	Immature	≥ 1 biweekly s.c. vaccination[f]	• Patients who received WT-1 vaccine showed increased OS.
Takahashi et al., Cancer Immunol. Immunother. (34)	The above study group was extended to N = 240	II, III and IV[b]	WT-1 and/or MUC-1 peptide	Immature	≥ 5 biweekly s.c. vaccinations[f]	• Having a DTH response was correlated with increased survival. • No difference in OS between patients vaccinated with WT-1 DCs and patients vaccinated with other DC vaccines was determined.
Ge et al., BMC Cancer (35)	N = 15	I, II and IIIA	Survivin and MUC-1 peptides[g]	Partly mature[h]	3 weekly i.v. vaccinations	• Circulating Tregs were significantly decreased 2 weeks after vaccination. • Improved quality of live was reported.
Li et al., Oncol. Lett. (36)	N = 16	I, II and III	MAGE-A3 and Survivin peptides	Mature	16 rounds of two monthly i.d. vaccinations	• All patients showed a DTH response. • In 15 out of 16 patients, tumor-specific IFN-γ T cell responses were increased.

Studies are displayed in order of publication (old to new). [a]*Patients without response to first-line treatment or who declined first-line treatment were included,* [b]*DCs were HLA-DR*$^{+}$*CD86*$^{+}$*CD40*$^{+}$*CD80*^{low}*CD83*$^{-}$*CCR7*$^{-}$*,* [c]*No established maturation method was used and no data that showed the maturation status of the DCs was available,* [d]*When patients showed no disease progression after vaccination, 1 boost vaccination per 4 weeks was administered,* [e]*Patients who had inoperable tumors or relapsed quick after surgery,* [f]*When patients showed no disease progression, vaccination was repeated.* [g]*DCs were also incubated with inhibitors of suppressor of cytokine signalling 1 (SOCS1),* [h]*DCs were HLA-DR*$^{+}$*CD80*$^{+}$*CD83*$^{+}$*CD86*$^{+}$*CD40*$^{+}$*CD14*$^{-}$*CCR7*$^{-}$*; MUC-1, Mucin-1; CEA, carcinoembryonic antigen; i.d., intradermal; s.c., subcutaneous; DTH, delayed-type hypersensitivity; WT-1, Wilms' tumor protein 1; MAGE-1, melanoma-associated antigen 1; her-2/neu, human epidermal growth receptor 2; i.v., intravenous; IFN, interferon; Tregs, T regulatory cells; IL-2, interleukin 2; COX-2, cyclo-oxygenase 2; TLR-7, Toll-like receptor 7; DFS, disease-free survival.*

towards the vaccine or by performing a delayed-type hypersensitivity (DTH) test. The principle of a positive DTH test is that if T cells are activated by DC vaccination, this DC vaccine will be recognized upon injection in the skin. This will cause a local immune reaction resulting in erythema. Most studies initially confirmed expression of their used tumor antigen in the tumor or used autologous tumor lysate for DC priming. Interestingly, vaccine-specific immunological responses

were demonstrated in most studies after vaccination. However, this induced tumor-specific immune response was almost never linked to a radiological response or improved survival.

DC VACCINATION COMBINATION THERAPIES FOR NSCLC

Investigators have also been focusing on the effect of combining DC vaccination with other therapies. For instance, chemotherapy and radiotherapy are hypothesised to enhance anti-tumor immunity and could therefore synergize with immunotherapy. A well-described effect of chemotherapy and radiotherapy is immunogenic cell death of cancer cells, exposing high levels of tumor antigen and DAMP molecules to immune cells in the TME (37–39). The superior immune-activating ability that chemotherapy and radiotherapy induce in cancer cells is highlighted in DC vaccination studies of cancer mouse models exploiting this strategy. In these studies, DC vaccination of DCs loaded with radiation-treated or chemotherapy-treated cancer cells, resulted in reduced tumor volume compared to mice vaccinated with DCs loaded with untreated tumor cells (40–43). Moreover, chemotherapy and radiotherapy were reported to stimulate human leukocyte antigen I (HLA-I) expression of the tumor, making tumor cells more sensitive to cytotoxic killing by $CD8^+$ T cells (38). It is important to define the optimal dose of chemotherapy or radiotherapy for combination treatment with immunotherapy, as high doses of chemo- and radiotherapy can induce cell death of immune cells as well (37). The synergistic effect of chemotherapy to DC vaccination was recently validated in a human melanoma study (44). For NSCLC, this synergistic effect of both chemotherapy and radiotherapy with DC vaccination was confirmed in mouse models (45–47). In human NSCLC, only one study examined the combination of chemotherapy with DC monotherapy, but many studies investigated the combined effect of chemo- and/or radiotherapy, DC vaccination and cytokine-induced killer cells (CIK) (**Table 2**) (48, 50–55). CIK cells consist of a heterogeneous group of T cells, NK cells, and NKT cells. CIK cells are derived from autologous PBMCs, activated and expanded *ex vivo* under influence of anti-CD3 and cytokines, such as interferon γ (IFN-γ) and interleukin 2 (IL-2) (56). CIK therapy was shown to be safe and had a response rate of 39% in various tumors. Moreover, CIK treatment was associated with increased survival (57). Co-culture of CIK cells and DCs enhanced cytolytic function of CIK cells and increased both IL-12 secretion by DCs and levels of immunostimulatory receptors on DCs as well as CIK cells (58).

Importantly, all studies showed that combination therapy was safe and well-tolerated. From all studies that combined DC-based therapy with radio- and/or chemotherapy in NSCLC, four out of seven demonstrated improved OS in the combination therapy group compared to radiotherapy or chemotherapy alone. Two studies of combined DC-CIK therapy that showed no differences in OS between groups, did show improved disease-free survival (DFS) in the combination therapy group. Unfortunately, the study investigating the effect of chemotherapy and DC vaccination alone included no control group to compare treatment efficiency or clinical outcome.

In addition to chemotherapy and radiotherapy, one study investigated the combination of DC-CIK and the EGFR tyrosine kinase inhibitor erlotinib in patients with advanced stage NSCLC (**Table 2**) (49). This study demonstrated increased progression-free survival (PFS) in the combination therapy group, while OS did not differ between the groups. This synergistic effect is particularly interesting considering that EGFR-mutated NSCLC is insensitive to anti-PD-1/anti-PD-L1 therapy (15, 59, 60). Erlotinib is normally not combined with other systemic treatment, because it shows no benefit in survival to monotherapy, while toxicity potentially increases (61, 62).

Whereas the current DC-based monotherapy studies could not show clinical benefit in NSCLC patients, combinations with chemotherapy, radiotherapy, and targeted therapy showed to improve clinical outcome. However, in almost all combination studies CIK cells were administered simultaneously with the DC vaccine. Hence, whether the observed clinical advantage of combination therapy over the standard therapy is an effect of the DC vaccine, the CIK cells, or the combination of both cannot be discerned from those studies.

A combination of therapies that was not studied in NSCLC before is DC-based therapy and other immunotherapy, such as ICIs. Several studies have pointed out that when there is no anti-tumor immune response, 'releasing the brakes' by checkpoint inhibition will not lead to improved clinical results. Hence, a combination with an immune strategy that actively induces an anti-tumor immune response might improve therapy response rate (63). Vice versa, therapies that actively stimulate the immune response, often result in increased expression of immune checkpoint molecules and might therefore also benefit from a combination treatment with ICIs. In addition, in single-cell RNA sequencing data from NSCLC tissue a mature DC subset with high expression of regulatory molecules, such as PD-L1, was identified which could be targeted by anti-PD-L1 therapy (64).

The potential synergistic effect of ICIs and DC vaccination is currently examined in advanced stage melanoma patients. Accordingly, two studies showed that a combination strategy of anti-CTLA-4 and DC vaccination resulted in an improved clinical response compared to similar cohorts that received anti-CTLA-4 treatment alone, without causing additional toxicity (65–68). In addition, ICI therapy was shown to be effective in advanced stage melanoma patients with recurrent disease after adjuvant DC vaccination (69). Until date, no results are available of studies that examine whether the synergistic effect of combined ICI and DC-based therapy also applies for NSCLC.

FROM PERIPHERAL IMMUNE ACTIVATION TOWARDS A LOCAL RESPONSE

An important question is whether the current administration route for DC vaccination in NSCLC can induce a tumor-specific

TABLE 2 | Characteristics of studies in which DC vaccination combination therapy was performed in NSCLC.

Ref.	Subject number	Clinical stage	Antigen source	DC maturation status	Type and regimen of DC administration	Most important results after vaccination
Zhong et al., Cancer Immunol. Immunother. (48**)**	N = 28 (DC-CIK + chemotherapy = 14; chemotherapy = 14)	III and IV	CEA peptide	Immature	• All patients received 4 cycles of vinorelbine with cisplatin chemotherapy. • The DC-CIK + chemotherapy group in addition received 4 monthly cycles of i.v. DC-CIK vaccinations.	• Patients in the DC-CIK + chemotherapy group demonstrated significantly increased PFS compared to the chemotherapy only group. • There was no difference between 1-, 2-, and 5-year OS between the different groups.
Shi et al., J. Immunother. (49**)**	N = 54 (erlotinib + DC-CIK = 27, erlotinib = 27)	III and IV	Tumor lysate	Immature	• All patients received erlotinib. • The DC-CIK + erlotinib group in addition received 4 s.c. DC vaccinations and 5 i.v. CIK vaccinations within the erlotinib treatment. • Patients received treatment cycles until disease progression or withdrawal from the study (≥ 2 cycles).	• Circulating CD4 T cells, CD8 T cells and the CD4/CD8 ratio were significantly increased after erlotinib + DC-CIK treatment, while there were no differences in these parameters in the erlotinib only group.. • PFS was significantly increased in the DC-CIK + erlotinib group compared to the erlotinib only group. • There was no difference in OS between both treatment groups.
Hu et al., Med. Oncol. (50**)**	N = 27[a]	III and IV	Tumor lysate	Immature	• Patients received pemetrexed chemotherapy followed by i.d. DC vaccination at day 12. • Patients received multiple rounds of DC vaccination until disease progression (≥ 2 cycles) or up to a maximum of 6 rounds.	• Primary endpoint was safety and combination therapy was shown safe. • No clinical nor immunological effect could be determined, since no control group was available.
Zhao et al., Exp. Ther. Med. (51**)**	N = 157 (DC-CIK + chemotherapy = 79; chemotherapy = 78)	IIIA	–	Immature	• All patients received surgery. • Chemotherapy consisted of four cycles of gemcitabine and cisplatin. • 2 i.v. DC-CIK vaccinations were administered after the second cycle and after the fourth cycle of chemotherapy in the DC-CIK + chemotherapy group.	• The 3-year cumulative recurrence rate was significantly reduced in the DC-CIK + chemotherapy group. • The 3-year cumulative survival was significantly increased in the DC-CIK + chemotherapy group.
Zhu et al., Genet. Mol. Res. (52**)**	N = 65 (DC-CIK + radio-/ chemotherapy = 30; radio-/ chemotherapy = 35)	IIIB	-	Unknown[b]	• All patients received 4 cycles of docetaxel and cisplatin chemotherapy combined with a total dose of 60-70 Gy radiotherapy. • The DC-CIK + radio/chemotherapy group received 4 rounds of 2 or 3 i.v. DC-CIK vaccinations in between the chemo- and radiotherapy cycles.	• Patients in the DC-CIK + radio/ chemotherapy group demonstrated significantly increased CD3 and CD4 T cells 4 weeks after treatment. This difference was not observed in the radio/ chemotherapy group only. • Patients in the DC-CIK + radio/ chemotherapy group demonstrated significantly increased 6-months and 12-months OS compared to the radio-/ chemotherapy alone group.
Zhang et al., Oncol. Lett. (53**)**	N = 507 (DC-CIK + standard therapy = 99; standard therapy = 408)	III and IV	NSCLC cell line lysate	Unknown[b]	• Standard therapy consisted of surgery, chemotherapy, and radiotherapy. • DCs were administered i.v. once a week for 3 weeks. • In the first week of treatment, patients received i.v. CIK vaccinations once a day for 4 days. After 3 weeks, patients received i.d. DC vaccinations once a week for 3 weeks.	• 59 out of 97 patients from the combination therapy group demonstrated a DTH response (the control group was not tested). • Patients who received DC-CIK showed significantly improved survival compared to patients who received standard therapy.
Zhang et al., Radiot. Oncol. (54**)**	N = 82 (DC-CIK + radiotherapy = 21; radiotherapy = 61)	III and IV	MUC-1 peptide	Unknown[b]	• All patients received a total dose of 60-66 Gy radiotherapy. • The DC-CIK + radiotherapy group received 4 s.c. DC vaccinations and 4 i.v. CIK vaccinations between radiotherapy fractions.	• Peripheral blood of before and after treatment was available for 20 patients. No differences in circulating CD8 T cells, CD4 T cells and NK cells were observed between before and after treatment in both treatment groups.

(Continued)

TABLE 2 | Continued

Ref.	Subject number	Clinical stage	Antigen source	DC maturation status	Type and regimen of DC administration	Most important results after vaccination
						• Patients in the DC-CIK + radiotherapy group showed a significantly increased PFS compared to the radiotherapy only group. • No difference in OS between both treatment groups was observed.
Zhao et al., Clin. Transl. Oncol. (55)	N = 135 (DC-CIK = 45; chemotherapy = 40; DC-CIK + chemotherapy = 50	III and IV	–	Partly mature[c]	• Chemotherapy consisted of pemetrexed or docetaxel. • DC-CIK was administered i.v. daily for 3 days. • Patients of all groups received ≥ 2 rounds of treatment.	• In multivariate analysis, combination therapy of DC-CIK and chemotherapy was an independent prognostic factor for increased 1-year PFS and OS. • There was no difference in 1-year OS between the DC-CIK only and the chemotherapy only group.

Studies are displayed in order of publication (old to new). DC-CIK therapy or DCs for DC vaccination were derived from autologous PBMCs. [a]All patients failed gefitinib or erlotinib maintenance therapy. [b]No established maturation method was used and no data that showed the maturation status of the DCs was available, [c]showed a $CD80^+CD86^+$ population > 80% in their vaccine; SCC, squamous cell carcinoma; TNF-α, tumor necrosis factor α; CEA, carcinoembryonic antigen; i.v., intravenous; s.c., subcutaneous; MUC-1, Mucin-1; PFS, progression-free survival.

immune response in the lungs. The most promising results from human DC-based therapies are achieved in melanoma. In these trials, DCs were injected in the skin and migrated to cutaneous lymph nodes in which maturated DCs can initiate an anti-tumor immune response. For melanoma, this is often in close proximity to the tumor. For NSCLC this same route of administration is chosen, although the tumor is located at a large distance from the cutaneous lymph nodes. This difference in environment of T cell activation might lead to a decreased amount of T cells that reach the tumor. This is illustrated in a pancreatic cancer mouse model in which intraperitoneal administration of a DC vaccine suppressed tumor growth and inhibited tumor progression to a larger extent compared to subcutaneous injections of the same DC vaccine (70). It might therefore be interesting to study the effect of DC-based therapy that is administered into the local lymph nodes. Although this is more invasive, it might induce a more locally effective anti-tumor immune response.

The local environment during T cell activation might not be the only element causing the suggested suboptimal lung T cell infiltration. In an inflammation mouse model, it was shown, that after local immunization with the immunogenic ovalbumin protein (OVA), DCs isolated from lung-draining mediastinal lymph nodes induced increased lung homing of CD4 T cells compared to DCs isolated from muscle-draining inguinal lymph nodes (71). The authors linked this increased ability to induce lung-homing of CD4 T cells to a $CD24^+$ DC subset that is highly expressed in the mediastinal lymph node compared to the inguinal lymph node. To induce a local immune reaction in the lung, it therefore seems pivotal to specifically target this DC subset. Interestingly, in another inflammation mouse model it was demonstrated that this specific DC subset is probably not induced in the lung-draining lymph node itself, but rather in the lungs before lymph node migration (72). This is illustrated by an experiment in which DCs isolated from lung tissue, lung-draining lymph nodes, and other lymph nodes received antigen and were co-cultured with T cells *ex vivo*. DCs isolated from lung were superior at inducing lung-homing T cells, while there was no difference between DCs originating from lung-draining lymph nodes and other lymph nodes. This finding that lung-derived DCs induced superior homing of T cells to the lung was also confirmed in another mouse model of viral infection. In this model, mice were intranasally challenged with viral particles after which DCs were isolated from both lung and lung-draining lymph nodes at multiple time points after infection. Their results demonstrate that at 30 minutes after infection lung DCs were superior at inducing T cell homing compared to DCs originating from lung-draining lymph nodes, while at 24 hours after infection this difference was abolished. Since transport of soluble antigen from the lung towards the local lymph node already occurs within a few minutes after infection, this experiment validates that DCs that migrated from the lung are the main stimulants for lung-homing T cells. Although these are all pre-clinical data, a lung-derived DC subset paramount for optimal local tumor-specific T cell immune response might also be present in humans. DCs used for DC vaccination in NSCLC are not lung-derived. Therefore, means to equip DCs with an optimal capacity to induce lung-homing T cells should be developed.

IN VIVO TARGETING OF DCS

The clinical trials using DC-based therapy in NSCLC were performed with DCs that were earlier isolated from the patient's peripheral blood, after which the DC vaccine was finalized in the lab. This procedure to construct DC vaccines has disadvantages: it is demanding for patients, laborious and expensive. For that reason, targeting DCs *in vivo* is a promising approach. Moreover, specific DC subsets in the tumor could be directly targeted when a monoclonal antibody directed against specific endocytic DC receptors would be used as guide for antigen delivery. For example, C-type lectin domain containing 9A (CLEC9A) on conventional type 1 DCs (cDC1s). Since CLEC9A is involved in cross-presentation, this specific

targeting could also skew the antigen-processing towards this direction (73). This strategy of specific DC-targeting is not yet performed in humans, but some mouse studies show promising results.

In melanoma mouse models, receptors targeting among others, CLEC9A, CD11c, and DEC-205 (receptor on circulating DCs of mice and human, involved in cross-presentation) were bound to OVA (74–77). With concurrent activation, such as Polyinosinic:polycytidylic acid (poly I:C) and anti-CD40, the administered tumor antigen-receptor complex was shown to elicit effective immune responses and inhibit tumor growth. Hence, in the study using DEC-205 it was even demonstrated that *in vivo* vaccination showed larger inhibition of tumor growth compared to vaccination with *ex vivo* spleen-derived DCs which were also primed with OVA and maturated using anti-CD40 (74).

In the previous examples, antigens were chemically conjugated to a monoclonal antibody, or the DNA sequence of the antigen was genetically fused to the monoclonal antibody, while DC activation signals were injected separately. Currently, however, many studies focus on more complex manners of antigen delivery *in vivo* that could improve antigen uptake and DC activation efficiency. Examples are lipid vesicles or nanoparticles with surface-bound DC targeting receptors, and containing tumor antigens and DC activation signals (78). An important advantage of this technique is that the maturation signals are selectively delivered to the DCs. This is important because maturation molecules have been shown to have a tumor-supporting function when binding to other cells in the TME (79–81).

There are also studies that only focus on *in vivo* DC activation, without loading the DC with antigen. In a lymphoma mouse model, it is shown that intratumoral injection of TriMix mRNA, encoding costimulatory molecules CD70, CD40 ligand, and constitutively active toll-like receptor 4 (TLR4), induces systemic tumor-specific T cell responses independent of the co-delivery of tumor antigen (82). Moreover, in animal cancer models they show increased survival after injection of TriMix mRNA. The uptake of Trimix mRNA relies on the ability of DCs to rapidly and selectively internalize free RNA and avoids off-target effects. These results suggest that tumor-infiltrating DCs may have acquired antigen, and that the problem in their malfunctioning phenotype is rather the lack of sufficient activation signals in their surroundings.

CONCLUSION

Nowadays, DC-based therapy in NSCLC is still at a developmental stage. The DC vaccination studies performed are all early-phase studies that demonstrated low toxicity of the treatment, but were underpowered to show clinical benefit. However, most of these clinical trials showed that DC vaccination can induce the desired immune response. The latter highlights the potential of DC-based therapy in NSCLC and encourages further research that could advance the peripheral immunological effect to a radiological response or improved survival. In particular, studies that examine whether the anti-tumor immune response in peripheral blood or skin could also be induced in the tumor environment could provide more insight. The immune-activating ability of DC vaccination and the low toxicity of treatment make this therapy an excellent candidate for combination with other anti-cancer treatment. Clinical success of combination therapies is illustrated by the results of combination studies of chemotherapy and/or radiotherapy and even targeted therapy with DC-CIK vaccination in NSCLC. Likewise, studies in melanoma demonstrated the synergizing effect of DC-based therapy and ICIs. Especially this latter combination with ICIs, which inhibit the immunosuppressive TME, could allow the optimal immune-activating potential of DC based therapy to be revealed in NSCLC.

AUTHOR CONTRIBUTIONS

IAEvdH wrote the manuscript. GFG and BP wrote and reviewed the manuscript. IJMdV and MMvdH reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Dendritic Cell-Mediated Regulation of Liver Ischemia-Reperfusion Injury and Liver Transplant Rejection

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Liver allograft recipients are more likely to develop transplantation tolerance than those that receive other types of organ graft. Experimental studies suggest that immune cells and other non-parenchymal cells in the unique liver microenvironment play critical roles in promoting liver tolerogenicity. Of these, liver interstitial dendritic cells (DCs) are heterogeneous, innate immune cells that appear to play pivotal roles in the instigation, integration and regulation of inflammatory responses after liver transplantation. Interstitial liver DCs (recruited *in situ* or derived from circulating precursors) have been implicated in regulation of both ischemia/reperfusion injury (IRI) and anti-donor immunity. Thus, livers transplanted from mice constitutively lacking DCs into syngeneic, wild-type recipients, display increased tissue injury, indicating a protective role of liver-resident donor DCs against transplant IRI. Also, donor DC depletion before transplant prevents mouse spontaneous liver allograft tolerance across major histocompatibility complex (MHC) barriers. On the other hand, mouse liver graft-infiltrating host DCs that acquire donor MHC antigen *via* "cross-dressing", regulate anti-donor T cell reactivity in association with exhaustion of graft-infiltrating T cells and promote allograft tolerance. In an early phase clinical trial, infusion of donor-derived regulatory DCs (DCreg) before living donor liver transplantation can induce alterations in host T cell populations that may be conducive to attenuation of anti-donor immune reactivity. We discuss the role of DCs in regulation of warm and liver transplant IRI and the induction of liver allograft tolerance. We also address design of cell therapies using DCreg to reduce the immunosuppressive drug burden and promote clinical liver allograft tolerance.

Keywords: liver, dendritic cells, ischemia-reperfusion injury, immune regulation, transplant tolerance

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INTRODUCTION

Dendritic Cell Biology and Diversity

Our understanding of DC development and function is based largely on extensive studies in mouse models and human *in vitro* systems. DCs are heterogeneous innate immune cells that link innate and adaptive immunity (1). They are subdivided into conventional DCs (cDCs) that acquire, process and present antigen (Ag), and non-conventional plasmacytoid DCs (pDCs) that produce

type-1 interferon (IFN) following viral stimulation. While indispensable for antiviral immunity, pDCs also promote or regulate other inflammatory/immune responses (2, 3). cDCs and pDCs arise from a common bone marrow precursor in a fms-like tyrosine kinase 3 ligand (Flt3L)-dependent manner (4–7). Mouse cDCs are further divided into two subsets,- cDC1 (CD11c+,CD103+,CD11b-) and cDC2 (CD11c+,CD103-, CD11b+) that differentiate under the influence of IFN regulatory factor (IRF) 8 and IRF4, respectively. Mouse pDCs are CD11c+, CD103-, CD11b-,B220+,Gr-1+, sialic acid-binding immunoglobulin-like lectin (Siglec) H+ (5, 6, 8–11). Human cDCs express high levels of CD11c and are subdivided into CD1c+ (blood DC Ag (BDCA)1+), CD1b+, CD11b+, CD14+ DC that promote T helper (Th)17 cells and correspond broadly to mouse cDC2s, versus CD141+ (BDCA3+) DC that promote Th1 cell responses and Ag cross-priming to CD8+ T cells, corresponding to mouse DC1s. Human pDCs express CD123 (IL-3R), CD14 and CD303 (BDCA2) and potently produce type-1 IFN. Each DC subset in mice and humans develops under the control of a specific repertoire of transcription factors involving differential levels of IRF8 and IRF4 expression (12–16).

Liver Dendritic Cells

Multiple DC subsets have been identified in the liver, although their relative abundance differs from that in peripheral blood and secondary lymphoid tissue (17–21). cDC1, cDC2 and pDCs and their functional relevance in the steady-state and liver disease have been reviewed (21). Improved understanding of liver DC heterogeneity and function in mice and humans is required to further elucidate their roles and for design of DC-directed therapeutic intervention in liver injury, transplantation and other liver disorders. Recently, single cell RNA sequencing (seq) analysis has been used to quantify liver DC subsets (cDC1, cDC2 and pDC) and to define signatures of DC-T cell interactions in draining lymph nodes under healthy conditions and in liver disease (22). Mouse liver DC heterogeneity has also been described using cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) (23). The phenotype and function of liver interstitial DCs is influenced by the hepatic microenvironment that promotes their inherent tolerogenicity in the healthy steady-state (24–26). Thus, *via* their production of macrophage colony-stimulating factor and other soluble and cell-cell contact factors, liver stromal cells induce regulatory cDCs that secrete high levels of IL-10 and nitric oxide (NO), but little IL-12 and inhibit T cell proliferative responses/induce activated T cell apoptosis (24, 27, 28). Exposure to gut-derived pathogen-associated microbial products e.g. bacterial lipopolysaccharide (LPS) inhibits liver cDC or pDC maturation by stimulating IL-6- signal transducer and activator of transcription 3 (STAT3) activity that upregulates expression of interleukin-1 receptor-associated kinase M (IRAK-M), an inhibitor of Toll-like receptor (TLR) signaling (29). This phenomenon, referred to as endotoxin tolerance (30), extends to several TLRs (cross-tolerance), as well as to TLRs and ischemic injury. By contrast, exposure to LPS stimulates secretion of IL-10 and IL-27 by liver cDCs that can then expand regulatory T cells (Tregs) (31, 32).

Liver cDCs also express comparatively low levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules (33), but comparatively high levels of the T cell co-inhibitory molecule programed death ligand -1 (PD-L1). Compared with lymphoid tissue DCs, they also express high levels of the ectoenzyme CD39 (34) that degrades adenosine triphosphate to adenosine, and the immunoreceptor transmembrane adaptor protein DNAX activating protein of 12 kDa (DAP12) that regulates their maturation (35). Like liver cDCs, liver pDCs express comparatively high levels of DAP12 and high PD-L1:CD80/86 ratios (36, 37) and secrete IL-10. Thus, liver DCs are refractory to stimulation with microbial products and express gene products that undermine effector T cell responses, but promote Tregs (38).

We discuss below reported roles of liver DCs in regulation of liver IRI and immune responses to liver allografts. We also consider how regulatory DC (DCreg) therapy is being introduced in clinical trials to ascertain its potential to promote reduced dependency on/withdrawal of immunosuppression (IS) in liver transplantation.

LIVER ISCHEMIA REPERFUSION INJURY (IRI)

Graft IRI remains an understudied area in transplantation, despite its clinical significance. Hepatocellular damage associated with liver removal, storage and engraftment is critical to primary graft non-function or late dysfunction and may promote acute and chronic rejection and graft loss (39–41). IRI is a complex process that occurs when hypoxic tissue damage is increased by the inflammatory pathways that are activated during the return of blood flow and oxygen delivery, that combines elements of "warm" and "cold" injury (39, 42). Warm IRI is dominated by liver macrophage-derived cytotoxic molecule-mediated hepatocellular damage. Cold IRI, that occurs during ex-vivo organ preservation, is dominated by damage to liver sinusoidal endothelial cells (SECs) and disruption of the microcirculation (39, 43, 44).

Liver DCs and Regulation of Liver IRI

Regulatory properties of liver DCs have been described in both liver warm and cold (transplant) IRI in the mouse (**Table 1**).

Liver Warm IRI

Loi et al. (45) reported that liver DCs isolated after hepatic warm IR exhibited a more mature surface phenotype than those from uninjured liver, but preferentially produced the anti-inflammatory cytokines IL-10 and transforming growth factor b that might inhibit T cell and natural killer (NK) cell stimulation after IRI. It was also shown (46) that targeted deletion of cDCs by injecting CD11c-diptheria toxin (DT) receptor mice with DT 12–18 hours prior to I/R increased liver injury. Moreover, cDCs reduced liver IRI by secreting IL-10 that inhibited IL-6, tumor necrosis factor (TNF) and reactive oxygen species production by inflammatory monocytes recruited to the liver. More recent work (49) indicates that signaling *via* the prostaglandin E receptor EP3 in DCs promotes liver repair after warm IR by inducing IL-13-

TABLE 1 | Regulation of liver ischemia-reperfusion injury by intra-hepatic dendritic cells.

Model (species)	Observation	Protective or deleterious effect of DCs	Reference
Warm IR (mouse)	IR results in enhanced expression of anti-inflammatory cytokines (IL-10; TGFb) but reduced expression of IL-12 by liver DCs	Protective	Loi et al. (45)
Warm IR (mouse)	Targeted deletion of cDCs increases liver injury; cDCs reduce liver IRI *via* IL-10 secretion	Protective	Bamboat et al. (46)
Warm IR (mouse)	VitD analogue administration promotes tolerogenic DCs and attenuates liver injury; interruption of DC-T cell interaction (with anti-CD44) increases proinflammatory DC maturation and enhances tissue damage	Protective	Funken et al. (47)
Warm IR (mouse)	Adoptive transfer of WT but not DAP12-/- DCs reduces liver IRI in DAP12-/- mice *	Protective	Nakao et al. (48)
Warm IR (mouse)	EP3-expressing DCs orchestrate the pro-reparative environment during liver repair after hepatic IR	Protective	Nakamoto et al. (49)
Warm IR (mouse)	Increasing liver DCs in WT but not in TLR4 KO mice promotes liver injury	Deleterious	Tsung et al. (50)
Warm IR (mouse)	Liver injury less in DC-deficient (Flt3L -/-) mice	Deleterious	Zhang et al. (51)
Warm IR (mouse)	Blockade of TIM-4 on hepatic DCs ameliorates liver injury	Deleterious	Li et al. (52)
Warm IR (mouse)	pDC-depleted mice display reduced liver IR injury	Deleterious	Castellaneta et al. (53)
Liver transplant IR (mouse)	Livers from DC-deficient (Flt3L -/-) donors exhibit enhanced injury	Protective	Zhang et al. (51)
Liver transplant IR (mouse)	Portal venous delivery of WT but not CD39-/- liver cDCs to donor livers protects against graft injury	Protective	Yoshida et al. (34)

cDC, conventional DC; DAP12, DNAX activating protein of 12kDa; EP3, E prostanoid receptor 3; Flt3L, fms-like tyrosine kinase 3 ligand; pDC, plasmacytoid DC; TGFb, transforming growth factor b; TIM-4, T cell Ig domain and mucin domain 4; vitD, vitamin D; WT, wild-type.
**DAP12-/- mice exhibit enhanced liver warm IRI compared with WT mice.*

mediated switching of macrophages from pro-inflammatory to IL-10-producing, reparative cells. Vitamin D analogue administration promotes regulatory DCs and attenuates liver warm IRI, whereas interruption of DC-T cell interaction enhances proinflammatory DC maturation and tissue damage (47). Adoptive transfer of wild-type (WT) but not DAP12-/- cDCs reduces warm liver IRI in DAP12-/- mice that exhibit enhanced tissue injury compared with WT animals (48). Taken together, these findings suggest a protective role for DCs in warm liver IRI.

Other data however, conflict with this view. Fms-like tyrosine kinase 3 ligand (Flt3L) is a potent, endogenous DC poietin. Flt3L KO mice exhibit profound reductions in mDC and pDC in liver and lymphoid tissues (51, 54, 55). In these Flt3L KO mice (51), warm liver IR results in reduced hepatic injury, with less polymorphonuclear cell infiltration compared with WT animals. Absence of hepatic interstitial DC in this study also induces less upregulation of inflammatory cytokine and chemokine (TNFa, CCL2 and CXCL2) gene expression in the liver. Moreover, adoptive transfer of splenic or hepatic WT DC into Flt3L KO or WT mice increases hepatic warm IR injury. TIM-4 (T cell immunoglobulin domain and mucin domain containing 4) expression by liver cDC has been reported to play an important role in mouse segmental warm IRI (52); its blockade by anti-TIM-4 antibody reduces liver injury and inflammatory cytokine production and facilitates induction of Foxp3+ Tregs, suggesting a potential therapeutic approach. Thus, in contrast to the protective roles of liver DC described above, these findings suggest injurious effects of DC in liver warm IRI (51). In another report (50), increasing cDCs in the liver by GM-CSF hydrodynamic transfection increased liver injury after warm IR in WT but not TLR4 KO mice. With respect to liver pDCs, mice depleted of these cells using anti-pDC Ag (PDCA)-1 antibody failed to upregulate hepatic IFNa and exhibited reduced levels of hepatic IL-6, TNFa and liver injury after warm IR compared with WT controls (53).

Thus, while reports using different experimental approaches suggest both protective and deleterious effects of liver DCs in liver warm IRI, the balance of reports indicate protective properties of these cells in mouse models (53, 56–59). Further studies, taking into account liver DC heterogeneity and focused on the role of specific hepatic DC subsets, as well as the release of small extracellular vesicles with proinflammatory versus reparative properties by these cells during warm IRI (60, 61), may help elucidate these conflicting observations. Depending on microenvironmental conditions, complement system activators and inhibitors may also influence the differentiation/function of DC subsets towards immunogenicity or tolerance (62) and may be worthy of further investigation in the context of liver DCs their regulation of hepatic inflammatory responses.

Liver Transplant IRI

Several molecules have been implicated in regulation of liver transplant (cold) IRI by DCs. Absence of CD39 in liver grafts enhances cold IRI, associated with higher levels of pro-inflammatory cytokines (IL-6, TNFa, monocyte chemoattractant protein-1, IL-12p40), compared to WT donors. In addition, these CD39-/- allografts express higher levels of DC maturation markers

(CD80, CD86, MHC II) and lower levels of coinhibitory PD-L1. Moreover, adoptive transfer of WT liver mDCs exerts a protective effect against transplant-induced liver IRI, that is not achieved by CD39-/- liver mDC infusion (34, 63).

When Flt3L KO donor livers (lacking interstitial DCs) are transplanted into syngeneic WT mice with 24 hours of cold ischemia, the grafts show dramatically increased IR injury, with enhanced alanine transaminase levels, hepatic necrosis and neutrophil infiltration, indicating a protective role of liver-resident DC in WT livers (51). Thus, from the limited studies undertaken to date, liver DCs appear to have a protective role against liver transplant IRI in mice.

LIVER TRANSPLANT TOLERANCE

The liver is considered a tolerogenic environment, as evidenced by oral tolerance, portal venous tolerance, the ability of adeno-associated viral gene therapy to induce systemic tolerance to a transgene (64), metastasis of tumors to the liver and, in animals, acceptance of liver allografts across MHC barriers, without IS therapy (65–69). Within the liver microenvironment, multiple parenchymal and non-parenchymal cell populations (including DCs, Kupffer cells, SECs and stellate cells) express gene products e.g. indoleamine dioxygenase, arginase and PD-L1, that suppress inflammatory and immune-mediated responses (70). DCs express human leukocyte Ig-like receptor B (LILRB) family members, ligation of which renders DCs tolerogenic, leading in turn, to suppression of T cell responses (71) and immune tolerance in humanized mice. Since LILRB family members are considered receptors for HLA-G, that can be produced by liver cells (hepatocytes, liver stem/progenitor cells and biliary epithelial cells) (27, 72–74), this may potentially be an additional mechanism of immune regulation within the liver environment. The liver is also considered a site in which T cells activated therein exhibit defective cytotoxic function (75), and a site of increased T cell apoptosis (76). Potential mechanisms that may mediate liver transplant tolerance have been reviewed recently (26, 77).

Liver DCs and Regulation of the Balance Between Liver Transplant Tolerance and Rejection

Hematopoietic progenitors within the liver are programed to differentiate into DCregs with comparatively low MHC II and T cell costimulatory molecule expression and high IL-10 but low IL-12 secretion. Both liver cDCs and pDCs only weakly stimulate allogeneic T cell proliferation and can promote activated T cell hyporesponsiveness/apoptosis and Tregs (32, 78–80). Together with other liver NPCs, liver DCreg appear to play key roles in the induction of liver transplant tolerance (24); reviewed in (25, 26, 70, 81). The properties of mouse and human hepatic DCs that may promote regulation of alloreactive T cell responses/tolerance induction are summarized in **Table 2**.

In the liver, the coinhibitory molecule PD-L1 is expressed constitutively by DCs, Kupffer cells and SECs (91, 92). PD-L1 also can be up-regulated on both NPCs and hepatocytes following inflammatory stimulation (55, 93–95). It has been reported that transplantation of mouse liver allografts from PD-L1 KO donors, or blocking of PD-1/PD-L1 interactions using anti-PD-L1 monoclonal antibody, results in acute liver allograft rejection. This is associated with increased graft $CD8^+$ T cell infiltration and FasL perforin, granzyme B, iNOS and OPN mRNA expression in the recipients (96).

Depletion of donor interstitial DCs before mouse liver transplantation using CD11c-diptheria toxin receptor (DTR) donor mice in which DCs are depleted by DT administration, prevents induction of spontaneous allograft tolerance (90). Moreover, donor-derived cDCs can be generated *ex vivo* from progenitors present in normal mouse liver. They can also be generated from lymphoid tissue of untreated recipients of liver but not heart allografts from the same donor strain that are rejected acutely (97). In addition, when adoptively transferred to prospective pancreatic islet allograft recipients, donor liver-derived cDCs prolong graft survival (38). Collectively, these and other observations have implicated donor-derived liver cDCs in the promotion of liver transplant tolerance (70).

Absence of the transmembrane adaptor protein DAP12 (that is constitutively expressed on liver DCs at higher levels that on secondary lymphoid tissue DCs) (35) in mouse liver allografts results in higher pro-inflammatory cytokine (IL-6, IL-12p40, IFNγ, and TNFα) gene expression within the graft, enhanced IFNγ production by graft-infiltrating $CD8^+$ T cells and systemic levels of IFNγ, but reduced incidences of $CD4^+Foxp3^+$ cells, associated with acute graft rejection (98).

Non-lymphoid tissue pDCs, such as those that reside in the airways, gut and liver, play a significant role in regulating mucosal immunity and are critical for the development of tolerance to inhaled or ingested/dietary Ags (99). The liver is a site of oral Ag presentation and compared to secondary lymphoid tissue, is comparatively rich in pDCs (18) that appear to rapidly induce anergy or deletion of Ag-specific T cells (37, 79). We have reported (37) that hepatic pDCs of donor origin, that express high levels of DAP12, triggering receptor of myeloid cells 2 (TREM2) and high ratios of T cell coinhibitory PD-L1:costimulatory CD86 compared with secondary lymphoid tissue pDCs, play a key role in attenuating graft-infiltrating T effector cell responses, enhancing $Foxp3^+$ Tregs, and promoting spontaneous acceptance of mouse liver allografts.

Recently, we have also examined the role of graft-infiltrating DCs in regulation of mouse spontaneous liver transplant tolerance. The phenomenon of plasma membrane fragment transfer or "cross-dressing" between leukocytes was reported in 1999 (100). It has been postulated that molecules acquired by acceptor APCs during this process influence subsequent T cell responses. Several recent publications (101–103) have drawn attention to an important role of cross-dressed DCs (CD-DCs) in rejection of experimental heart, kidney and skin transplants. However, our recent novel findings (104) suggest that graft-infiltrating host cDCs that acquire donor MHC Ag shortly after liver transplantation *via* cross-dressing, regulate anti-donor T cell responses and promote allograft tolerance.

TABLE 2 | Properties of hepatic DCs that promote their immune regulatory function and may contribute to tolerance induction.

DC subset (species)	Property	Effect	Reference(s)
cDCs			
Mouse	Low MHC class II and costimulatory molecule expression	Infusion into prospective pancreatic islet allograft recipients prolongs graft survival	Rastellini et al. (38)
Mouse	Low MHC class II and co-stimulatory molecule expression	Infusion induces IL-10-producing cells in allogeneic host lymphoid tissue	Khanna et al. (33)
Mouse	Low MHC class II and costimulatory molecule expression	Systemic administration induces donor-specific T cell hyporesponsiveness in a sponge allograft model	Chiang et al. (82)
Mouse	Low TLR4 expression	Induction of alloAg-specific T cell hyporesponsiveness following LPS stimulation	Dr Creus et al. (83)
Mouse (also pDCs)	Gut-derived bacterial products inhibit liver DC maturation by stimulating IL-6-STAT3 activity that upregulates IRAK-M expression	Higher maturation marker expression by IL-6 -/- liver cDCs and pDCs	Lunz et al. (29)
Human	Production of IL-10 but not IL-12p70, even after TLR4 stimulation	Poor ability to stimulate allogeneic T cell proliferation; stimulation of IL-10 but suppression of IFNg production by T cells	Goddard et al. (84); Kwekkeboom et al. (85)
Human	Liver perfusate DCs exhibit low costimulatory molecule expression and produce high IL-10 levels in response to TLR4 ligation	Impaired T cell stimulatory capacity compared with skin or secondary lymphoid tissue DCs	Bosma et al. (86)
Mouse	Periportal and sinusoidal liver DCs loaded with Ag in the portal vein	Induce Th2 responses in the liver, enhance apoptosis of Ag-specific T cells and prevent hepatic injury caused by Th1 cells.	Watanabe et al. (87)
Mouse	Reduced costimulatory molecule and IL-12 expression induced by contact with sinusoidal endothelial cells	Impaired ability to prime naïve CD8 T cells	Schildberg et al. (88)
Mouse	IL-10 production; low Delta 4/Jagged 1 Notch ligand ratio	Skew towards allogeneic Th2 cell differentiation; CD4 T cell apoptosis; poor T cell allostimulatory activity associated with Treg function	Tokita et al. (79)
Mouse	Liver stromal cell-induced DCs secrete high IL-10/low IL-12; produce PGE2	Inhibit T cell proliferation/induce apoptosis of activated T cells; alleviate autoimmune hepatitis	Xia et al. (24)
Human	Liver stromal cells impair DC differentiation and maturation (role for PGE2)	Impaired ability to induce T cell proliferation	Bruno et al. (27)
Mouse	Liver stroma induces regulatory DCs producing NO and IL-10	Inhibition of CD8 T cell proliferation	Wang et al. (28)
Human	Secrete substantial IL-10 upon TLR4 ligation	Generate more suppressive Tregs than blood DCs *via* an IL-10-dependent mechanism	Bamboat et al. (32)
Mouse	LPS-stimulated liver DCs secrete IL-10 and IL-27	Induce T cell hyporesponsiveness, associated with selective Treg expansion	Chen et al. (31)
Mouse & Human	Liver DCs with low lipid levels	Induce regulatory T cells, anergy to cancer, and oral tolerance	Ibrahim et al. (89)
Mouse	Comparatively high cell surface CD39 expression	Hyporesponsiveness to ATP; reduces responses to TLR4 ligation and proinflammatory and immunostimulatory activity	Yoshida et al. (34)
Mouse	Absence of cDCs in donor liver allografts	Acute liver allograft rejection	Yokota et al. (90)
pDCs			
Mouse	IL-27 production and STAT3-dependent IL-27-induced PD-L1 expression	Promote Tregs; adoptive transfer suppresses DTH responses	Matta et al. (36)
Mouse	Express high levels of DAP12/TREM2 and high PD-L1: CD86 ratios	Potently suppress allogeneic T cell proliferation; pDC-depleted donor livers rejected acutely and Treg and exhausted CD8 T cells in grafts reduced; Treg in LNs reduced	Nakano et al. (37)

Ag, antigen; ATP, adenosine triphosphate; DAP12, DNAX-activating protein of 12 kDa; DTH, delayed- type hypersensitivity; IRAK-M, interleukin-1 receptor-associated kinase M; LPS, lipopolysaccharide; LN, lymph node; MHC, major histocompatibility complex; PD-L1, programed death ligand-1; PGE2, prostaglandin E2; STAT3, signaling transducer of activated T cells; Th, T helper; TLR, Toll-like receptor; TREM2, triggering receptor of myeloid cells 2.

Therapeutic Application of DCregs in Clinical Liver Transplantation

Properties of DCregs and approaches to promoting their tolerogenic functions in transplantation are depicted in **Figure 1**. Following the initial observation that infusion of liver-derived cDCs, one week before transplant, could promote subsequent donor-strain allograft survival in mice (38), many rodent studies have confirmed the ability of donor-derived DCs (cDCs or pDCs) with immunoregulatory properties to enhance organ allograft survival and donor-specific tolerance (105–108). In addition, the safety and efficacy of donor-derived mDCs in prolonging MHC mis-matched renal allograft survival has been demonstrated in a clinically-relevant nonhuman primate model using a minimal IS drug regimen (109). These promising findings have provided a rationale and justification for an early phase (phase 1/2; open label, non-controlled, non-randomized) clinical trial of donor-derived DCregs in an IS drug withdrawal study in adult living donor liver transplant (LDLT) patients at the University of Pittsburgh (110).

This first-in-human study commenced in late 2017 (NCT 03164265),- donor-derived DCregs generated from circulating blood monocytes have been infused into 15 prospective liver

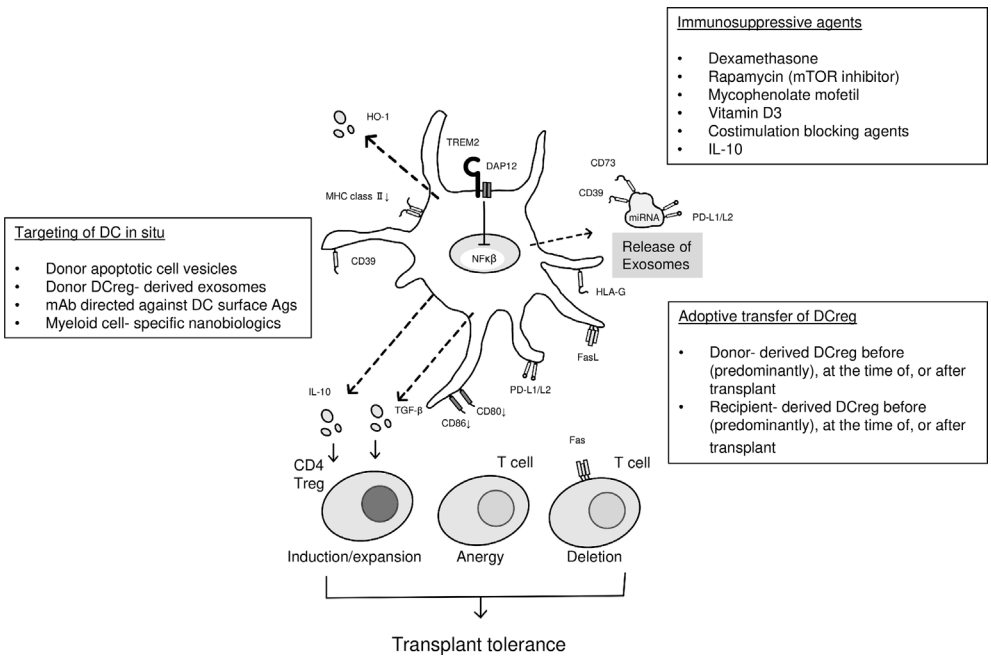

FIGURE 1 | DCreg and promotion of their function. Center, DCreg showing cell membrane-expressed and secreted/molecules and released small extracellular vesicles (exosomes) that can regulate T cell responses and immune reactivity; left panel, approaches to targeting of DCreg *in situ*; upper right panel, use of immunosuppressive agents that promote DC tolerogenicity; lower right panel, adoptive transfer of DCreg in transplant recipients. DAP12, DNAX activating protein of 12 kDa; HO-1, hemoxygenase-1; PD-L1/2, programed death ligand1/2; miRNA, microRNA; TGFB, transforming growth factor beta; TREM2; triggering receptor expressed on myeloid cells 2.

transplant patients, once only, one week before transplantation, together with a half dose of mycophenolate mofetil (MMF) to minimize any low potential risk of host sensitization. The DCregs that are infused exhibit a tolerogenic gene transcriptional profile, high cell surface PD-L1 to CD86 ratios, secrete high levels of IL-10 but little of no IL-12 in response to TLR4- or and CD40 ligation, and only weakly stimulate proliferation of prospective graft recipient T cells (111). The dose of DCregs infused (2.5-10 x 10^6/kg body weight) is based on the dose range that proved safe and effective in the preceding NHP studies. Patients receive conventional, post-transplant IS with steroid, MMF and tacrolimus. A protocol biopsy is performed at 1 year and, if permissive, careful weaning of tacrolimus is undertaken. Target cell numbers have been achieved for each of the prospective liver recipients and no adverse events associated with DCreg infusion have been observed. In a second clinical IS drug withdrawal study, also being performed in LDLT patients at the University of Pittsburgh (NCT04208919), a single donor-derived DCreg infusion is being administered to stable graft recipients enrolled 1-3 years post-transplant following biopsy confirmation of the absence of rejection. In addition to determining the safety of the infused DCreg product, an important objective of these studies is to determine preliminary efficacy of DCreg infusion in achieving complete IS drug withdrawal. Currently, drug withdrawal can only be achieved in 10-15% of adult liver allograft recipients in the first 2 years post-transplant (112).

Mechanistic Studies

The initial trial of donor-derived DCregs in LDLT is being accompanied by mechanistic studies aimed at understanding the *in vivo* fate of the donor-derived DCregs and the influence of their infusion on host anti-donor immune reactivity. Following DCreg infusion one week before transplant, intact donor DCregs can be detected in host peripheral blood shortly after completion of the infusion by discriminatory MHC class I staining and flow cytometric analysis. By 3 days post infusion, no intact donor DCregs can be detected. However, in several HLA-A2 negative graft recipients given HLA-A2 positive donor cells, transiently elevated levels of both donor HLA and immunoregulatory PD-L1, CD39 and CD73 could be detected in circulating small extracellular vesicles (sEVs) (111). At the same time, flow and advanced image stream analysis revealed "cross-dressing" of host DCs in the peripheral blood and in host lymph nodes obtained at the time of surgery, before graft implantation. PD-L1 co-localization with donor HLA was observed at significantly higher levels than with recipient HLA (111). These findings resemble our observations (113) of graft-infiltrating host DCs cross-dressed with donor MHC class I Ag and co-expressing high levels of PD-L1 in mouse liver allograft recipients that accept liver allografts without IS therapy. These cross-dressed recipient DCs marked inhibited anti-donor T cell proliferation *ex vivo*. Our observations in patients also resemble the identification of circulating host APCs cross-dressed with donor MHC Ag in human liver allograft recipients early and

transiently after transplantation (114). In our studies, between the time of donor DCreg infusion and liver transplantation, memory $CD8^+$ T cells expressing high levels of the transcription factors T-bet and Eomesodermin ($T\text{-}bet^{hi}Eomes^{hi}$) decreased, whereas regulatory ($CD25^{hi}CD127^{-}Foxp3^{+}$):$T\text{-}bet^{hi}Eomes^{hi}$ $CD8^+$ T cell ratios increased. Although the number of observations is small, this increase appeared to be associated with the incidence of cross-dressed DCs observed in the circulation. Thus, it appears that donor-derived DCreg infusion in prospective liver transplant recipients may induce systemic changes in host APCs and T cells that may be conducive to modulated anti-donor immune T cells responses at the time of transplantation. We postulate that the composition (quality) of sEVs rather than the density (quantity) of peptide MHC-expressing sEVs on cross-dressed DC may play an important role in the induction of peripheral tolerance.

CONCLUSIONS

Liver interstitial DCs appear to play important roles in the regulation of hepatic IRI and other inflammatory responses within the liver environment. Donor-derived DCs and more recently, graft-infiltrating host DCs that have acquired intact donor MHC Ag *via* cross-dressing, have been implicated in the promotion of spontaneous liver transplant tolerance in the mouse. Demonstrations that adoptive transfer of donor-derived DCregs can prolong organ transplant survival and tolerance in preclinical models has led to clinical testing of DCregs for promotion of transplant tolerance in human liver transplantation. These studies are accompanied by mechanistic investigations designed to enhance insight into the influence of these cells on host anti-donor immune reactivity.

AUTHOR CONTRIBUTIONS

RN: drafting of the manuscript. LT, DG, CM, and DM: critical review of the manuscript. AT: concept, design and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Plasmacytoid Dendritic Cells as a New Therapeutic Target for Autoimmune Pancreatitis and IgG4-Related Disease

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Although plasmacytoid dendritic cells (pDCs) able to produce large amounts of type 1 interferons (IFN-I) play beneficial roles in host defense against viral infections, excessive activation of pDCs, followed by robust production of IFN-I, causes autoimmune disorders including systemic lupus erythematosus (SLE) and psoriasis. Autoimmune pancreatitis (AIP), which is recognized as a pancreatic manifestation of systemic immunoglobulin G4-related disease (IgG4-RD), is a chronic fibroinflammatory disorder driven by autoimmunity. IgG4-RD is a multi-organ autoimmune disorder characterized by elevated serum concentrations of IgG4 antibody and infiltration of IgG4-expressing plasmacytes in the affected organs. Although the immunopathogenesis of IgG4-RD and AIP has been poorly elucidated, recently, we found that activation of pDCs mediates the development of murine experimental AIP and human AIP/IgG4-RD *via* the production of IFN-I and interleukin-33 (IL-33). Depletion of pDCs or neutralization of signaling pathways mediated by IFN-I and IL-33 efficiently inhibited the development of experimental AIP. Furthermore, enhanced expression of IFN-I and IL-33 was observed in the pancreas and serum of human AIP/IgG4-RD. Thus, AIP and IgG4-RD share their immunopathogenesis with SLE and psoriasis because in all these conditions, IFN-I production by pDCs contributes to the pathogenesis. Because the enhanced production of IFN-I and IL-33 by pDCs promotes chronic inflammation and fibrosis characteristic for AIP and IgG4-RD, neutralization of IFN-I and IL-33 could be a new therapeutic option for these disorders. In this Mini Review, we discuss the pathogenic roles played by the pDC-IFN-I-IL-33 axis and the development of a new treatment targeting this axis in AIP and IgG4-RD.

Keywords: autoimmune pancreatitis, IgG4-related disease, plasmacytoid dendritic cells, interferon-I, interleukin-33

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Abbreviations: pDCs, Plasmacytoid dendritic cells; IFN-I, Interferon-I; SLE, Systemic lupus erythematosus; AIP, Autoimmune pancreatitis; IgG4-RD, Immunoglobulin G4-related disease; IL-33, Interleukin-33; TLR, Toll-like receptor; MyD88, Myeloid differentiation primary response protein 88; IRAK4, Interleukin-1-receptor-associated kinase 4; TRAF, Tumor-necrosis factor receptor-associated factor; IKKα, Inhibitor of NF-κB kinase α; IRF, Interferon regulatory factor; STAT, Signal transduction and activator of transcription; NETs, Neutrophil extracellular traps; HMGB1, High-mobility group box 1; DT, Diphtheria toxin; poly (I:C), polyinosinic:polycytidylic acid; PSL, Prednisolone; T1D, Type 1 diabetes; Treg, Regulatory T cell.

INTRODUCTION

Plasmacytoid dendritic cells (pDCs) were initially identified over two decades ago as a unique subset of dendritic cells that can produce abundant quantities of type 1 interferons (IFN-I) (1). Although pDCs constitute a very small percentage of human and murine immune cells (2), this cell type is a major cellular source of IFN-I and plays critical roles in host defense against microbial infection. This idea is supported by the findings that mice lacking pDCs or those treated with a pDC-depleting antibody (Ab) exhibit defective IFN-I responses (3). Activation of pDCs, followed by enhanced IFN-I production, is essential for the initiation of innate immune responses against viral infections (3, 4). Recent reports indicate that during infection with severe acute respiratory syndrome coronavirus 2, which has recently caused a pandemic worldwide, host immune defenses involve pDC activation (5). However, excessive production of IFN-I by pDCs also underlies the immunopathogenesis of a broad range of autoimmune disorders (6). Typical autoimmune diseases driven by the activation of pDCs include systemic lupus erythematosus (SLE) (7, 8), psoriasis (9), and type 1 diabetes (T1D) (10, 11). A recent clinical trial, in which patients with active SLE were successfully treated with biologics targeting IFN-I, verified the pathogenic roles of IFN-I produced by pDCs (12–14).

Type 1 autoimmune pancreatitis (AIP), which is recognized as a pancreatic manifestation of systemic immunoglobulin G4-related disease (IgG4-RD), is a chronic fibroinflammatory disorder of the pancreas (15–18). In this article, type 1 AIP is hereafter referred to as 'AIP'. IgG4-RD and AIP are newly established multi-organ autoimmune disorders characterized by elevated serum concentrations of IgG4 Ab and infiltration of IgG4-expressing plasmacytes into the affected organs. Although some of the molecular mechanisms accounting for enhanced IgG4 Ab responses are being elucidated, their immunopathogenesis remains poorly understood. Recently, we found that activation of pDCs mediates the development of murine AIP and human AIP/IgG4-RD *via* the production of IFN-I and interleukin-33(IL-33) (17, 19–23). Depletion of pDCs or neutralization of signaling pathways mediated by IFN-I and IL-33 efficiently inhibited the development of experimental AIP. Furthermore, enhanced expression of IFN-I and IL-33 was observed in the pancreas and serum of patients with AIP and IgG4-RD. Moreover, AIP and IgG4-RD share the mechanism of their immunopathogenesis with other autoimmune diseases, including SLE and psoriasis, in that the autoimmunity is caused by IFN-I production by pDCs. However, the IL-33-mediated signaling pathway is activated only in AIP and IgG4-RD, but not in SLE or psoriasis. Given that enhanced production of IFN-I and IL-33 by pDCs promotes chronic inflammation and fibrosis, which are characteristic features of AIP and IgG4-RD, neutralization of IFN-I and IL-33 could be a new therapeutic option for these disorders. In this Mini Review, we discuss pathogenic roles played by the pDC-IFN-I-IL-33 axis and propose novel treatments targeting this axis in AIP and IgG4-RD.

IFN-I SIGNALING PATHWAYS IN PDCS

Innate immune responses initiated by Toll-like receptors (TLRs) are critical for host defense against pathogens (24). pDCs preferentially express endosomal TLR7 and TLR9, which detect single-stranded RNA and double-stranded DNA derived from bacteria and viruses (24). Innate immune responses mediated by TLR7 and TLR9 depend upon the activation of myeloid differentiation primary response protein 88 (MyD88). The interaction between TLR7/9 and MyD88 is followed by the activation of interleukin-1-receptor-associated kinase 4 (IRAK4) (24, 25). The kinase activity of IRAK4 mediates the formation of complexes consisting of IRAK1, tumor-necrosis factor receptor-associated factor 3 (TRAF3), TRAF6, inhibitor of NF-κB kinase α (IKKα), and interferon regulatory factor 7 (IRF7) (4, 24, 25). Formation of this complex leads to the nuclear translocation of IRF7, a critical transcription factor for the initial production of IFN-I in pDCs (24–26). IRF7, which is polyubiquitinated by TRAF6 after interaction with MyD88 (27), is the master regulator of IFN-I production. This idea is fully supported by studies showing that pDCs isolated from mice deficient in IRF7 or MyD88 exhibit defective IFN-I production upon stimulation with TLR9 ligands (28).

As mentioned above, sensing of single-stranded RNA and double-stranded DNA by endosomal TLR7 and TLR9 induces the activation of the MyD88-IRAK4-IRAK1-TRAF6-TRAF3-IKKα-IRF7 pathway, thereby leading to the initial production of IFN-I by pDCs (24–26). Although the initial production of IFN-I is low, IFN-I-mediated signaling pathways are augmented by the presence of a positive feedback loop (26, 29). IFN-I activates the cell surface IFN-I receptor followed by nuclear translocation of IFN-stimulated gene factor 3, which is composed of signal transduction and activator of transcription 1 (STAT1), STAT2, and IRF9 (26, 29), and promotes the transcription of IRF7 by binding to its putative promoter regions. The newly synthesized IRF7, in turn, leads to the amplification of IFN-I transcription. This positive feedback loop of IFN-I response is useful for the eradication of viruses and bacteria; however, it may also augment IFN-I responses associated with autoimmunity.

PATHOGENIC ROLES OF PDCS IN AUTOIMMUNE DISEASES

Although pDCs play beneficial roles in host defense against viral infections, excessive activation of pDCs, followed by robust production of IFN-I, causes autoimmune diseases. SLE is the most well-studied autoimmune disease the pathogenesis of which is significantly affected by the pDC-IFN-I axis (30). This notion is supported by the finding that elevated serum concentrations of IFN-I observed in patients with SLE correlate with both disease activity and severity (31). SLE is a chronic multi-organ disorder characterized by the production of Abs to self-nucleic acids and by the deposition of immune complexes (32, 33). In SLE, immune complexes composed of

self-nucleic acids and antinuclear Abs are efficiently taken up by cell surface Fc receptors and then delivered into the endosomal components of pDCs (3, 34, 35). Sensing of self-nucleic acids by endosomal TLR7 and TLR9 results in IFN-I production by pDCs. Thus, activation of TLR7 and/or TLR9 by self-nucleic acids is an indispensable step for pDC-mediated IFN-I responses in SLE.

Regarding the trigger for IFN-I production by pDCs, recent studies have highlighted the importance of neutrophil extracellular traps (NETs), web-like structures released by activated neutrophils (36–39). NETs, composed of chromatin DNA, oxidized mitochondrial DNA, antimicrobial peptides, and the high-mobility group box 1 (HMGB1) protein, can function as a trigger for excessive IFN-I secretion through the activation of TLR7 and/or TLR9 in pDCs of patients with SLE (36–39). In fact, increased formation of NETs and elevated concentrations of NET components, such as antimicrobial peptides and HMGB1, were observed in the serum of patients with SLE as compared with those in healthy controls (37). Thus, enhanced IFN-I production caused by NET-mediated TLRs activation underlies the immunopathogenesis of human SLE (**Figure 1**). As for transcription factors for IFN-I production involved in the immunopathogenesis of SLE, recent studies highlight the importance of IRF5 in parallel to IRF7 (40, 41). *IRF5* gene polymorphisms associated with SLE cause enhanced expression of IRF5 and hyperactivation of IRF5 underlies the immunopathogenesis of SLE through induction of IFN-I production (40, 41).

In line with human studies, experimental murine models of lupus provided evidence supporting the immunopathogenicity of pDCs. NZB and BXSB mice spontaneously develop murine lupus (30). Neutralization of the IFN-I receptor in BXSB mice and genetic deletion of the IFN-I receptor in NZB mice protected these mouse strains from the development of lupus, suggesting that spontaneous development of murine lupus required intact IFN-I signaling pathways (42, 43). Recent studies have successfully shown that pDC-mediated IFN-I responses cause experimental murine lupus. To investigate the specific contribution of pDCs in murine lupus, transgenic mice were created that expressed the diphtheria toxin (DT) receptor under the control of the highly specific human pDC *CLEC4C*/*BDCA2* promoter. Administration of DT before disease onset inhibited the development of lupus by selective systemic ablation of pDCs, which was accompanied by impaired expression of genes stimulated by IFN-I (44). Interestingly, these beneficial effects of transient pDC depletion were sustained even after pDC recovery, indicating crucial roles of pDC-mediated IFN-I responses in disease initiation (44). This idea was supported by another study in mice with impairment of pDC function caused by monoallelic deletion of the pDC-specific transcription factor E2-2. Sisirak et al. reported that impairment of pDC function resulted in the amelioration of murine lupus caused by the overexpression of TLR7 (45). These animal studies confirmed the concept that TLR-mediated IFN-I responses by pDCs play crucial roles in the development of both human SLE and murine lupus.

The pDC-IFN-I axis has been implicated in psoriasis development. Psoriasis is the most common autoimmune disease of the skin and is characterized by the infiltration of immune cells and hyperproliferation of keratinocytes (46). The accumulation of pDCs expressing IFN-I and IRF7 was much greater in the skin of patients with psoriasis than in those of

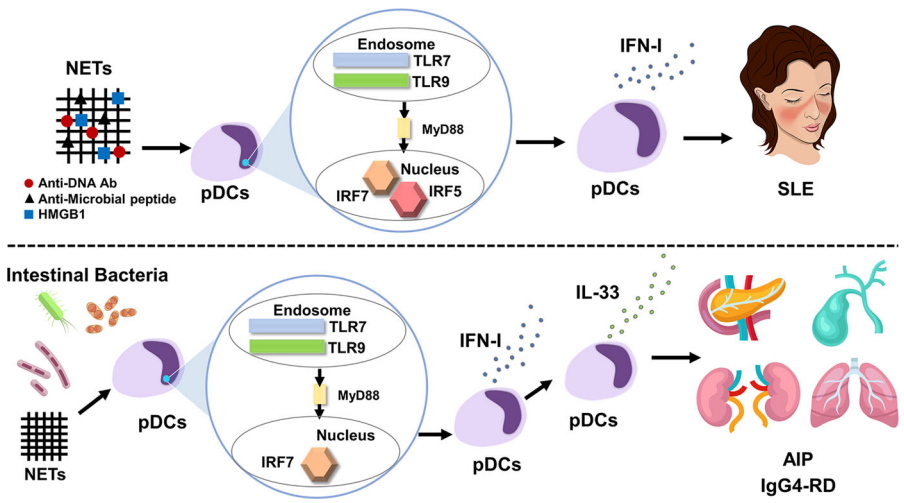

FIGURE 1 | IFN-I produced by plasmacytoid dendritic cells mediates systemic lupus erythematosus and autoimmune pancreatitis/IgG4-related disease. Neutrophil extracellular traps (NETs) activate plasmacytoid dendritic cells (pDCs) to produce large amounts of IFN-I. pDC-mediated IFN-I responses underlie the immunopathogenesis of systemic lupus erythematosus (SLE). Myeloid differentiation primary response protein 88 (MyD88)-dependent activation of Toll-like receptor 7 (TLR7) and TLR9 induces IFN-I production through nuclear translocation of interferon regulatory factor 5 (IRF5) and IRF7 (top panel). Intestinal dysbiosis and NETs activate pDCs to produce large amounts of IFN-I through nuclear translocation of IRF7, which, in turn, results in the enhanced production of IL-33 by pDCs. The pDC-IFN-I-IL-33 axis underlies the immunopathogenesis of autoimmune pancreatitis (AIP) and IgG4-related disease (IgG4-RD) (bottom panel).

healthy controls (9). Sensing of self-DNA coupled with antimicrobial peptides by TLR9 induces IFN-I production by pDCs residing in the skin of patients with psoriasis (47). Glitzner et al. directly addressed the role of pDCs in the development of experimental psoriasis by crossing $Jun^{f/f}JunB^{f/f}K5cre\text{-}ER^{T}$ mice with *BDCA2-DTR* mice to deplete pDCs (48). They found that the depletion of pDCs by DT injection resulted in the amelioration of experimental psoriasis, which was accompanied by downregulation of IL-23 expression (48). Thus, they provide evidence that activation of pDCs mediates psoriatic lesions by enhancing signaling pathways mediated not only by IFN-I but also by IL-23. The latter cytokine plays a crucial role in the development of psoriasis, as evidenced by the fact that biologics targeting IL-23 are very effective in patients with psoriasis (49). In addition to psoriasis, IFN-I production by pDCs plays pathogenic roles in the development of T1D (10, 11). In these studies, activation of pDCs by self DNA, DNA-specific IgG, and antimicrobial peptide induces IFN-I production through TLR9 in the pancreatic islets (10, 11).

PDCS AND AUTOIMMUNE PANCREATITIS/IGG4-RELATED DISEASE

Although AIP and IgG4-RD are characterized by enhanced adaptive immune responses that include the IgG4 Ab response, recent studies have shed light on the presence of innate immune responses as well. Repeated injection of polyinosinic: polycytidylic acid (poly (I:C)) into MRL/MpJ mice leads to the development of AIP, autoimmune sialadenitis, cholangitis, and glomerulonephritis, all of which are organ-specific manifestations of AIP and IgG4-RD (50). Extensive flow cytometry analyses performed using pancreatic immune cells found massive accumulation of pDCs in the pancreas of MRL/MpJ mice displaying AIP. Consistent with the pancreatic accumulation of pDCs, IFN-I expression was markedly enhanced in the pancreas of MRL/MpJ mice (19). The development of experimental AIP was dependent upon the activation of pDC-mediated IFN-I signaling pathways because the administration of pDC-depleting or IFN-I neutralizing Abs efficiently prevented the development of experimental AIP (19). Administration of the IRF7-specific siRNA almost completely prevented the development of experimental AIP through the downregulation of IFN-I expression, suggesting that experimental AIP required the nuclear translocation of IRF7 (21).

A specific type of fibrosis called storiform fibrosis is one of the characteristic pathological findings in AIP and IgG4-RD (15–18). IL-33 produced by pancreatic acinar cells induces chronic fibroinflammatory responses in experimental chronic alcoholic pancreatitis (51, 52). As in the case of chronic pancreatitis, the pancreatic expression of IL-33 is much greater in AIP mice than in normal mice (20). Interestingly, cell purification and cell depletion studies have revealed that pDCs are a cellular source of IL-33 (20). IL-33 that is produced by pDCs in an IFN-I dependent manner is necessary for the development of chronic fibroinflammatory responses in the pancreas, as is shown by the neutralization of IL-33-mediated signaling pathways and attenuation of experimental AIP by using an anti-ST2 Ab (20). Although, pro-IL-33 is proteolytically activated into a bioactive form by caspase-1, 3, 7, it remains unknown whether caspase-mediated processing is operating in IL-33 production by pDCs in AIP and IgG4-RD (53).

The clinical relevance of these data in experimental AIP has been verified in human samples from patients with AIP and IgG4-RD. pDCs expressing IRF7, IFN-I, and IL-33 accumulated in the pancreas of patients with AIP and IgG4-RD (19–21). Moreover, peripheral blood pDCs isolated from patients with AIP and IgG4-RD promoted IgG4 Ab production by naïve B cells present in the peripheral blood of healthy controls in an IFN-I-dependent and T cell-independent manner (19). Thus, these studies support the idea that pDC-mediated production of IFN-I and IL-33 underlies the immunopathogenesis of AIP and IgG4-RD. We recently identified serum concentrations of IFN-I and IL-33 as novel biomarkers for AIP and IgG4-RD (54). Serum concentrations of these two cytokines were much higher in patients with AIP and IgG4-RD than in those with chronic pancreatitis or healthy controls. In addition, the induction of remission by prednisolone (PSL) was associated with a marked reduction in serum concentrations of IFN-I and IL-33 in patients with AIP and IgG4-RD. Thus, IFN-I and IL-33 produced by pDCs are also useful as biomarkers in the clinical identification of patients with AIP and IgG4-RD.

NETs and intestinal dysbiosis have been implicated as possible triggers of pDC activation in AIP and IgG4-RD. NETs formation was confirmed in the pancreas of MRL/MpJ mice displaying AIP and in patients with AIP and IgG4-RD (19). In addition to NETs, intestinal dysbiosis also mediates pDC activation in experimental AIP. Bowel sterilization by antibiotics completely prevented the development of experimental AIP, which was accompanied by reduced activation of pDCs expressing IFN-I and IL-33 (22). Repeated injections of 10 μg and 100 μg poly (I:C) into MRL/MpJ mice induced mild and severe types of AIP, respectively (22). We took advantage of the relationship between poly (I:C) doses and AIP severity and then performed co-housing and fecal microbiota studies. As expected, mice treated with 10 μg of poly (I:C) developed a mild degree of AIP. Interestingly, mice treated with 10 μg of poly (I:C) developed severe AIP equivalent to that induced by injection of 100 μg of poly (I:C) upon co-housing with mice treated with 100 μg of poly (I:C) or when they were exposed to fecal microbiota from donor mice treated with 100 μg of poly (I:C) (22). Such development of severe AIP was associated with enhanced pancreatic accumulation of pDCs producing IFN-I and IL-33. Thus, these studies provide evidence that intestinal dysbiosis mediates the development of experimental AIP through the activation of pDCs (**Figure 1**). In line with the results of experimental AIP, alterations in fecal microbiota composition were observed in patients with AIP (23). Disappearance of *Klebsiella pneumoniae* was observed in the stool of two of three patients with AIP after successful induction of remission by PSL. Mice treated with 10 μg of poly (I:C) in combination with oral administration of heat-killed

K. pneumoniae developed more severe AIP as compared with the condition of mice treated with poly (I:C) or *K. pneumoniae* alone. Severe AIP induced by co-administration of poly (I:C) and *K. pneumoniae* was associated with increased accumulation of pDCs producing IFN-I and IL-33. Taken together, these findings suggest that intestinal dysbiosis mediates AIP through the activation of pDCs producing IFN-I and IL-33. However, it should be noted that cellular sources of IL-33 are not limited to pDCs (55–57). In particular, M2 macrophages present in the salivary glands have been identified as potent producers of IL-33. As for possible triggers for pDC activation in human AIP and IgG4-RD, NETs formation was observed in the pancreas of patients with IgG4-associated AIP (19). Moreover, intestinal dysbiosis was associated with the induction of remission in patients with AIP (23). Therefore, NETs and intestinal dysbiosis may function as possible triggers for pDC activation.

It is well established pDCs preferentially activate regulatory T cells (Tregs) (58, 59). If pDCs are abundant in the affected organs of AIP and IgG4-RD, then activation of Tregs is induced in the lesions of AIP and IgG4-RD. In fact, chronic inflammatory lesions of AIP and IgG4-RD are characterized by accumulation of Tregs (17).

PDCS AS A THERAPEUTIC TARGET IN AUTOIMMUNE DISEASES

Clinical success targeting the pDC-IFN-I axis in SLE led us to hypothesize that patients with AIP and IgG4-RD can be successfully treated by blocking this pathway (**Figure 2**). As in the case of SLE, neutralization of IFN-I by anifrolumab or sifalimumab may be effective in patients with AIP and IgG4-RD (12–14). In contrast to the case with SLE, IL-33 produced by pDCs can be another treatment target for AIP and IgG4-RD. Serum concentrations of IFN-I and IL-33 have been identified as novel biomarkers useful for the diagnosis and evaluation of disease activity in patients with AIP and IgG4-RD, whereas serum concentrations of the latter cytokine were comparable in patients with SLE and healthy controls (54, 60, 61). Therefore, an anti-ST2 Ab or etokimab (62) targeting IL-33-mediated signaling pathways might be a unique therapeutic option for patients with AIP and IgG4-RD. This notion is supported by the finding that the blockade of IL-33-mediated signaling pathways by anti-ST2 Ab prevented not only fibrogenesis but also inflammation in experimental AIP (20). Thus, biologics targeting IFN-I and IL-33 may be promising therapeutics in patients with AIP and IgG4-RD, as evidenced by the results of animal studies, in which the neutralization of IFN-I or IL-33-mediated signaling pathways by Abs efficiently prevented the development of experimental AIP.

In addition to the IFN-I-IL-33 axis, correction of intestinal dysbiosis by antibiotics or probiotics might be useful for the suppression of pDC activation. This idea is supported by the fact that bowel sterilization by a broad range of antibiotics almost completely prevented the development of experimental AIP (22). The intracellular signaling pathway involves the activation of endosomal TLR7 and TLR9, followed by nuclear translocation of IRF7, to initiate the transcription of IFN-I. In the case of SLE, chloroquine, a potent inhibitor of endosomal activation of TLR7 and TLR9, has been shown to offer a survival advantage (33). Moreover, mycophenolate mofetil, another inhibitor of IRF7 (63), is a first-line therapy in the management of lupus nephritis and cutaneous disease (33). Thus, chloroquine and mycophenolate mofetil may be effective for patients with AIP

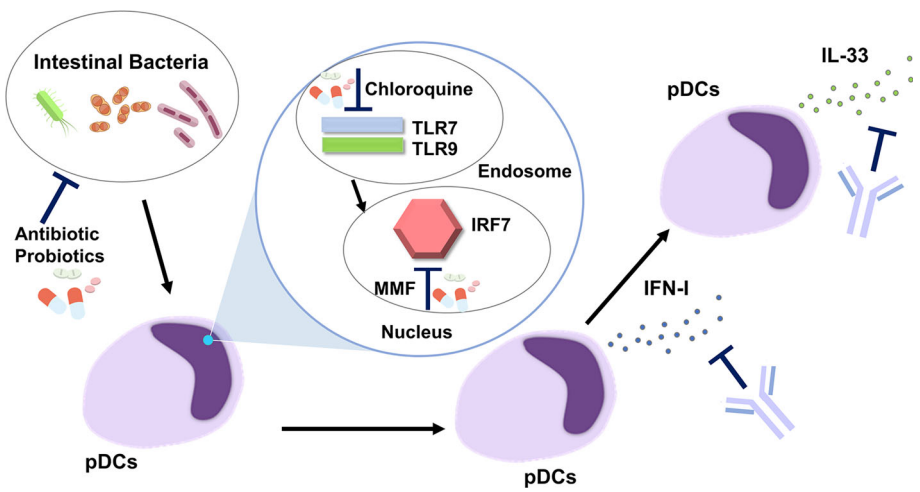

FIGURE 2 | Development of new treatments targeting the plasmacytoid dendritic cell-IFN-I-IL-33 axis in autoimmune pancreatitis and IgG4-related disease. Intestinal dysbiosis activates endosomal Toll-like receptor 7 (TLR7) and TLR9 followed by nuclear translocation of IFN regulatory factor 7 (IRF7). pDCs produce IFN-I through the nuclear translocation of IRF7. IL-33 is produced by pDCs in an IFN-I-dependent manner. Antibiotics and probiotics may be useful for correction of intestinal dysbiosis. Chloroquine inhibits the activation of endosomal TLR7 and TLR9. Mycophenolate mofetil (MMF) suppresses nuclear translocation of IRF7. IFN-I-mediated signaling pathways are efficiently inhibited by Abs against IFN-I or IFN-I receptor. An anti-ST2 Ab, neutralizing the IL-33 receptor, blocks IL-33-mediated signaling pathways.

and IgG4-RD as they would inhibit signaling pathways mediated by the activation of TLR7, TLR9, and IRF7.

In most cases of AIP and IgG4-RD, PSL is very effective for the induction of remission (15–18). It should be noted, however, that a significant fraction of patients with AIP and IgG4-RD experience repeated episodes of relapse, even upon standard treatment with PSL. Moreover, treatment with PSL can cause severe side effects such as diabetes mellitus, opportunistic infections, and osteoporosis. The new treatment targeting the pDC-IFN-I-IL-33 axis may be useful for such patients. Rituximab therapy is useful for patients with IgG4-RD (64). However, it is poorly understood whether induction of remission by rituximab is accompanied by reduction in IFN-I-IL-33 responses.

CONCLUSION

AIP and IgG4-RD are characterized by the activation of pDCs producing IFN-I and IL-33. Serum concentrations of IFN-I and IL-33 have been identified as novel biomarkers for AIP and IgG4-RD. Targeting the IFN-I-IL-33 axis in pDCs might constitute a successful approach to treat patients with AIP and IgG4-RD, especially those who suffer from repeated episodes of relapse even with PSL treatment or side effects associated with PSL.

AUTHOR CONTRIBUTIONS

KM and TW drafted the manuscript and prepared the figures. AH, TY, and KK reviewed the manuscript for its intellectual content. KM, TW, and MK were responsible for revising the manuscript. All authors contributed to the article and approved the submitted version.

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METHODS
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Optimal Isolation Protocols for Examining and Interrogating Mononuclear Phagocytes From Human Intestinal Tissue

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The human intestine contains numerous mononuclear phagocytes (MNP), including subsets of conventional dendritic cells (cDC), macrophages (Mf) and monocytes, each playing their own unique role within the intestinal immune system and homeostasis. The ability to isolate and interrogate MNPs from fresh human tissue is crucial if we are to understand the role of these cells in homeostasis, disease settings and immunotherapies. However, liberating these cells from tissue is problematic as many of the key surface identification markers they express are susceptible to enzymatic cleavage and they are highly susceptible to cell death. In addition, the extraction process triggers immunological activation/maturation which alters their functional phenotype. Identifying the evolving, complex and highly heterogenous repertoire of MNPs by flow cytometry therefore requires careful selection of digestive enzyme blends that liberate viable cells and preserve recognition epitopes involving careful selection of antibody clones to enable analysis and sorting for functional assays. Here we describe a method for the anatomical separation of mucosa and submucosa as well as isolating lymphoid follicles from human jejunum, ileum and colon. We also describe in detail the optimised enzyme digestion methods needed to acquire functionally immature and biologically functional intestinal MNPs. A comprehensive list of screened antibody clones is also presented which allows for the development of high parameter flow cytometry panels to discriminate all currently identified human tissue MNP subsets including pDCs, cDC1, cDC2 (langerin$^+$ and langerin$^-$), newly described DC3, monocytes, Mf1, Mf2, Mf3 and Mf4. We also present a novel method to account for autofluorescent signal from tissue macrophages. Finally,

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we demonstrate that these methods can successfully be used to sort functional, immature intestinal DCs that can be used for functional assays such as cytokine production assays.

Keywords: dendritic cells (DC), flow cytometry, human tissue, intestine, enzymatic digestion, macrophage – cell, mononuclear phagocyte cells (MNP)

INTRODUCTION

Intestinal mononuclear phagocytes (MNPs), specifically dendritic cells (DC), macrophages and monocytes, play a major role in maintaining tolerance to food-derived antigens and resident microbiota without compromising the hosts ability to respond to invading pathogens (1). DCs are professional antigen presenting cells (APC) capable of antigen uptake, processing and migration out of the tissue to draining lymph nodes where they present antigens and activate naïve T cells (2, 3). In contrast, macrophages perform this antigen presenting function weakly and do not readily migrate out of the tissue. Their primary function is to phagocytose and destroy invading pathogens as well as secrete a variety of immune-modulating cytokines (2, 3). Monocytes migrate into tissue from blood and differentiate into monocyte-derived macrophages or DCs. Embedded within these broad cell types are a diverse range of DC and macrophage subsets that each possess their own tissue-specific phenotype and function. Accurate identification of these subsets in the human intestine is required before we can understand their role in homeostasis and disease settings. Indeed, the misidentification of MNP subsets has caused much confusion in the literature (4–8).

Isolating MNPs from human tissues is a challenging task as the techniques used to liberate them can alter their phenotype and affect their viability (9). We have previously published optimised methods for isolating and interrogating immature DCs and macrophages from human abdominal skin *via* enzymatic digestion (9). However, skin and intestinal tissue have marked phenotypic, functional and structural differences which necessitates a modified approach to isolating MNPs. Recent literature has described a suite of consistent markers used to define all currently known subsets of human tissue DCs and macrophages (8–13). In human abdominal skin, these comprise $XCR1^+$ cDC1 (conventional DC), $CD1c^+$ cDC2 that includes langerin expressing and langerin negative populations and CD14-expressing cells including tissue-resident autofluorescent (AF) macrophages and monocyte-derived macrophages (13). In human intestinal tissue, three populations of DCs have been identified using CD103 and SIRPα (11, 12) and four intestinal $CD14^+$ macrophage populations (Mf1-4) have been identified using HLA-DR, CD14, CD11c and CD11b (10). More recently, high-resolution analyses have revealed a subpopulation within human blood cDC2s ($CD1c^+$ DCs) that expresses CD14 and a monocyte-like gene signature termed DC3s (14, 15). As defined by the literature, DC3s are $CD88^-$ $CD1c^+$ $CD163^+$ and express varying levels of CD14 (14, 15).

In this study, we present an intestinal tissue specific MNP isolation protocol to liberate high yields of viable, immature and biologically active MNPs from human intestinal jejunum, ileum and colon as well as terminal ileum biopsies. We also present techniques to anatomically separate the mucosa and submucosa, including their associated lymphoid follicles being Peyer's Patches in the small bowel and lymphoid aggregates in the large bowel, to better understand these distinct immune compartments. We emphasize the importance of carefully selecting antibodies that target the appropriate epitope post-digestion as well as markers that accurately define intestinal-derived MNPs according to the most recent and reliable literature. Further, we present a high-parameter flow cytometry gating strategy to identify all currently known human MNPs in human tissues. We also include a method for correcting AF spillover from tissue-resident macrophages which considerably improves the accuracy of measuring cell surface expression levels and correct MNP definition.

METHODS

Human Specimens

This study was approved by the Western Sydney Local Area Health District (WSLHD) Human Research Ethics Committee (HREC); reference number (4192) AU RED HREC/15 WMEAD/11. Large human intestinal specimens were taken with informed consent from patients undergoing surgery for intestinal cancer, 10-20 cm away from tumours, where present. Samples were processed within 2 hours of collection except for samples destined for cell sorting which were covered in Roswell Park Memorial Institute (RPMI) (Lonza, Switzerland) 1640 supplemented with 0.25% gentamycin and stored overnight at 4°C for processing the following morning.

Tissue Processing

Typical whole tissue intestinal specimens ranged in size from 5-40cm^2, with all data obtained with whole tissue specimens unless biopsies are stated. The muscularis externa was mechanically removed from the submucosa using curved surgical scissors and forceps. The tissue was then cut into approximately 25mm^2 pieces and incubated for 15 minutes twice in RPMI-1640 (Lonza) supplemented with 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich, Missouri, USA), 0.3% Dithiothreitol (DTT) (Sigma-Aldrich) and 2mM EDTA at 37°C (herein referred to as DTT treatment). The tissue was washed in Dulbecco's Phosphate Buffered Saline Mg^{++}/Ca^{++} free (DPBS) (Lonza) prior to incubation on a MACSmix Tube Rotator (Miltenyi Biotec, California, USA) 2-3 times in RPMI-1640 supplemented with 5μL/mL DNase I (Worthington Industries, New Jersey, USA) and 3mg/mL collagenase type IV (Worthington) for 30 min each time at 37°C. The supernatant was separated from undigested

intestinal tissue using a tea strainer. The cell suspension was passed through a 100μM cell strainer (Greiner Bio-One, North Carolina, USA) before being washed twice in DPBS. The single-cell suspension in RPMI-1640 was spun on a Ficoll-Paque PLUS (GE Healthcare, Illinois, USA) gradient and mononuclear cells were harvested from the RPMI-Ficoll interface. When required, the mucosa was anatomically separated from the submucosa using a dissection microscope and forceps. Lymphoid follicles present in the mucosa and submucosa were then dissected out using a scalpel. The mucosa was digested as above, with the submucosa not requiring the DTT treatment. For terminal ileum biopsies, a 10 min DTT treatment at 37°C was required. A single cell suspension from terminal ileum biopsies and isolated lymphoid follicles was generated by shaking them at 170rpm in a 37°C incubator in RPMI-1640 supplemented with 5μL/mL DNase I and 3mg/mL collagenase type IV for 45 min.

Flow Cytometry

Cells were labelled in aliquots of 1-3 x 10^6 cells per 100μL of DPBS. Non-viable cells were excluded by staining with Fixable Viability Stain 700 (BD Biosciences, New Jersey, USA). Cells were washed with FACS wash (1% FBS (v/v), 2 mM EDTA, 0.1% sodium azide (w/v) in PBS) and surface stained with the antibodies indicated in **Table 1** in 10μL Brilliant stain buffer plus (BD Biosciences) for 30 mins on ice. Cells were washed twice with FACS wash prior to resuspension in 100μL of CytoFix (BD Biosciences) for 15 mins at 4°C. Cells were washed and resuspended in 100μL of FACS wash. Flow cytometry was performed on an LSR Fortessa or FACSymphony flow cytometers (BD Biosciences) with BD Diva Software (V8.0) and data analysed on FlowJo (TreeStar V10.7.1).

Fluorescence Activated Cell Sorting

Single-cell suspensions to be sorted were enriched for $CD45^+$ cells using anti-CD45 microbeads (Miltenyi Biotec) as per the manufacturer's instructions. $CD45^+$ cells were stained with Live/Dead Near IR dead cell stain kit (Life Technologies, California, USA) in DPBS for 30 mins on ice, and washed with magnetic-activated cell sorting (MACS) wash [1% FBS (v/v), 2 mM EDTA in DPBS] before surface staining with antibodies indicated in **Table 2**. Cells were washed twice with MACS wash and filtered prior to sorting on a BD FACS Aria (130μM nozzle)

TABLE 1 | FACSymphony mononuclear phagocyte phenotyping panel.

Marker	Company	Clone	Fluorophore	Purpose	Concentration (μL/100μL)
Viability	BD Biosciences	–	FVS700	Live cells	1:10, 000
HLA-DR	BD Biosciences	G46-6	BUV395	Myeloid cells	0.5
CD45	BD Biosciences	HI30	BB755	Immune cells	0.5
CD3	BD Biosciences	UCTH1	BUV496	T lymphocytes	5
CD19	BioLegend	HIB19	BV750	B lymphocytes	5
CD14	BD Biosciences	M5E2	BUV737	Macrophages	2.5
Calprotectin	Invitrogen	MAC387	PE	Infiltrating monocytes	2.5
CD88	BioLegend	S5/1	PE Dazzle 594	Monocytes	0.5
CD1c	BD Biosciences	F10/21A3	BUV805	cDC2	2.5
FCϵR1α	Novus Bio	9E1	AF488	cDC2	1.5
FCϵR1α	BD Biosciences	AER-37	APC	cDC2	5
CD11b	BD Biosciences	ICRF44	BV711	MNPs	2
CD11c	BD Biosciences	B-Ly6	BB515	MNPs	1.5
CD123	BioLegend	6H6	PE/Cy5	pDCs	0.5
CD163	BioLegend	GHI/61	BV605	DC3	2.5
SIRPα	BioLegend	SE5A5	APC/Fire 750	DCs	2.5
Langerin	Miltenyi	MB22-9F5	PE-Vio770	cDC2	1.5
XCR1	BioLegend	S15046E	BV421	cDC1	4
CD141	Miltenyi	AD5-14H2	APC	cDC1	2.5

TABLE 2 | FACS Aria sort panel.

Marker	Company	Clone	Fluorophore	Purpose	Concentration (μL/100μL)
Viability	ThermoFisher	–	NIR	Live cells	1: 500
HLA-DR	Miltenyi	G46-6	PerCP	Myeloid cells	2
CD45	BD Biosciences	HI30	BV786	Immune cells	1
CD3	Miltenyi	REA613	APC-Vio770	T lymphocytes	2.5
CD19	Miltenyi	REA674	APC-Vio770	B lymphocytes	1
CD14	BD Biosciences	M5E2	BV421	Macrophages	2.5
CD1c	BD Biosciences	F10/21A3	PE	cDC2	2
CD11c	BD Biosciences	B-Ly6	PE-CF594	MNPs	1.5
Langerin	Miltenyi	MB22-9F5	PE-Vio770	cDC2	1.5
CD123	BD Biosciences	9F5	BV711	pDCs	1
XCR1	BioLegend	S15046E	APC	cDC1	4

(BD Biosciences), with sorted cells collected into 1.5 ml Eppendorf tubes (Sigma-Aldrich) containing RPMI-1640 supplemented with 10μM HEPES (Gibco, Massachusetts, USA), non-essential amino acids (Gibco), 1mM sodium pyruvate (Gibco) 50μM 2-mercaptoethanol (Gibco), 10μg/mL gentamycin (Gibco) and 10% (v/v) FBS (herein referred to as DC culture media).

Culturing of *Ex Vivo* Mononuclear Phagocytes

Following dead cell depletion (STEMCELL Technologies, Vancouver, Canada) as per the manufacturer's instructions and $CD45^+$ enrichment, 5 x 10^5 cells per condition were taken as a time 0-hour aliquot. All remaining cells were cultured at 1 x 10^6 cells/mL for 14 hours in DC culture media. Cells were then washed once in DPBS and stained with Fixable Viability Stain 700. Cells were washed with FACS wash and surface stained with antibodies indicated in **Table 1**, with drop-in CD54-PE (clone: HA58; 2.5μL/100μL), CD80-PE (clone: L307.4; 3μL/100μL), CD83-APC (clone: HB15e; 2.5μL/100μL) and CD86-APC (clone: 2331 (FUN-1); 2.5μL/100μL). Cells were washed twice with FACS wash prior to resuspension in 100μL of CytoFix (BD Biosciences) for 15 mins at 4°C. Cells were washed and resuspended in 100μL of FACS wash for acquisition.

Intracellular Cytokine Staining

Sorted intestinal-derived MNPs were cultured in DC culture media and stimulated with 1μg/mL of the TLR7/8 ligand, R848 (InvivoGen, California, USA). After 2 hours, 2.5μg/mL Brefeldin A (Sigma-Aldrich) was added to prevent cytokine secretion into the supernatant. After a total of 16 hours, cells were washed with DPBS and stained with Live/Dead Near IR dead cell stain kit. Cells were washed with FACS wash and resuspended in 100μL of CytoFix/CytoPerm (BD Biosciences) for 15 minutes at 22°C. Cells were washed twice with Perm Wash (1% FCS (v/v), 1% BSA (w/v), 0.1% saponin (w/v), 0.1% sodium azide (w/v) in PBS) and stained with anti-IL-6 APC (clone: MQ2-13A5; 2.5μL/100μL), anti-IL-23p40 PE (clone: C8.6; 0.25μL/100μL) and anti-TNF BV650 (clone: MAB11; 2.5μL/100μL) for 30 minutes at 22°C. Cells were washed and resuspended in 100μL of FACS wash prior to acquisition.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism v9.1.2 (San Diego, CA). A Student's paired t test (comparing 2 groups) or a Kruskal Wallis test with Dunn's multiple comparisons test (comparing more than 2 groups) was used.

RESULTS

Optimisation of Enzymatic Digestion Protocols for the Isolation and Interrogation of Intestinal Mononuclear Cells

We optimised a protocol for the mechanical separation and enzymatic digestion of human intestinal tissue to liberate MNPs. Enzymatic access to the mucosal tissue was increased by removing the underlying muscle layer and fine dicing the tissue with a scalpel. The epithelium and any remnant mucus were removed with DTT treatment prior to digestion with collagenase type IV at 37°C which we previously showed to the best collagenase blend for MNP tissue isolation (9) (**Figure 1A**). Using this method, we compared the yield and proportions of MNPs ($CD3^-$ $CD19^-$ HLA-DR^+ $CD45^+$) from sequential 30-minute collagenase digestions (**Figure 1B**). Digestions were performed in sequence rather than one extended digestion to both limit the exposure of the liberated cells to the enzyme, and to replenish the enzyme for optimal activity. The first digestion resulted in significantly lower yield and proportion of MNPs than the subsequent digestions, suggesting that 30 minutes was not long enough to liberate our cells of interest from whole tissue specimens. The second digestion provided the highest proportion of MNPs as well as the highest yield, in numbers suitable for phenotyping assays. (**Figures 1C, D**). A third digestion liberated additional cells which increased the total yield (**Figure 1C**), without compromising the proportion of HLA-DR^+ $CD45^+$ cells (**Figure 1D**) or their viability (**Figure 1E**). Two digestions were performed for phenotyping purposes, while three digestions were utilised when higher numbers of MNPs were required for cell sorting and functional assays.

Manual Separation of Intestinal Tissue Compartments

Intestinal mucosal tissue consists of multiple compartments that require separation to investigate their unique immune cell profiles (16). To achieve this, the mucosa and submucosa were mechanically separated along the biological border of the two layers, the muscularis mucosa, and lymphoid follicles were isolated prior to digestion (**Figure 2A**). This was performed under light microscopy without the need for tissue staining, with lymphoid follicles being visually identified and removed with a scalpel. The mucosa was digested as per the protocol for whole tissue (**Figure 1A**), with the submucosa digested without DTT treatment as it does not have an epithelial layer. Lymphoid follicles ($<2mm^2$), like biopsies ($<5mm^2$), are smaller than the diced pieces of whole tissue ($25mm^2$) and as such were fully digested in a shorter, single digestion. The lymphoid follicles as well as terminal ileum biopsies underwent collagenase type IV digestion for 45 min at 37°, with biopsies receiving a 10 min DTT treatment at 37°C before digestion as described in **Figure 2A**. Using tissue from 3 individual donors and a minimal staining panel we were able to show by flow cytometry that of the $CD45^+$ cells liberated, $CD3^+$ T cells, $CD19^+$ B cells and HLA-DR^+ MNPs were represented in different proportions in each compartment (**Figure 2B**), confirming that we can manually dissect intestinal tissue to enrich for specific tissue compartments using visual confirmation.

Accurate Identification of Tissue Dendritic Cell Subsets *via* Flow Cytometry

With the ever-changing classification of DCs (11, 12, 14, 17, 18), their identification by flow cytometry has required an increasing number of discriminatory markers. For tissue DCs this task is further complicated by the cleavage of surface identification markers that often occurs during enzymatic liberation. A subset of cDC2 that express FCεR1α have recently been described (14, 18).

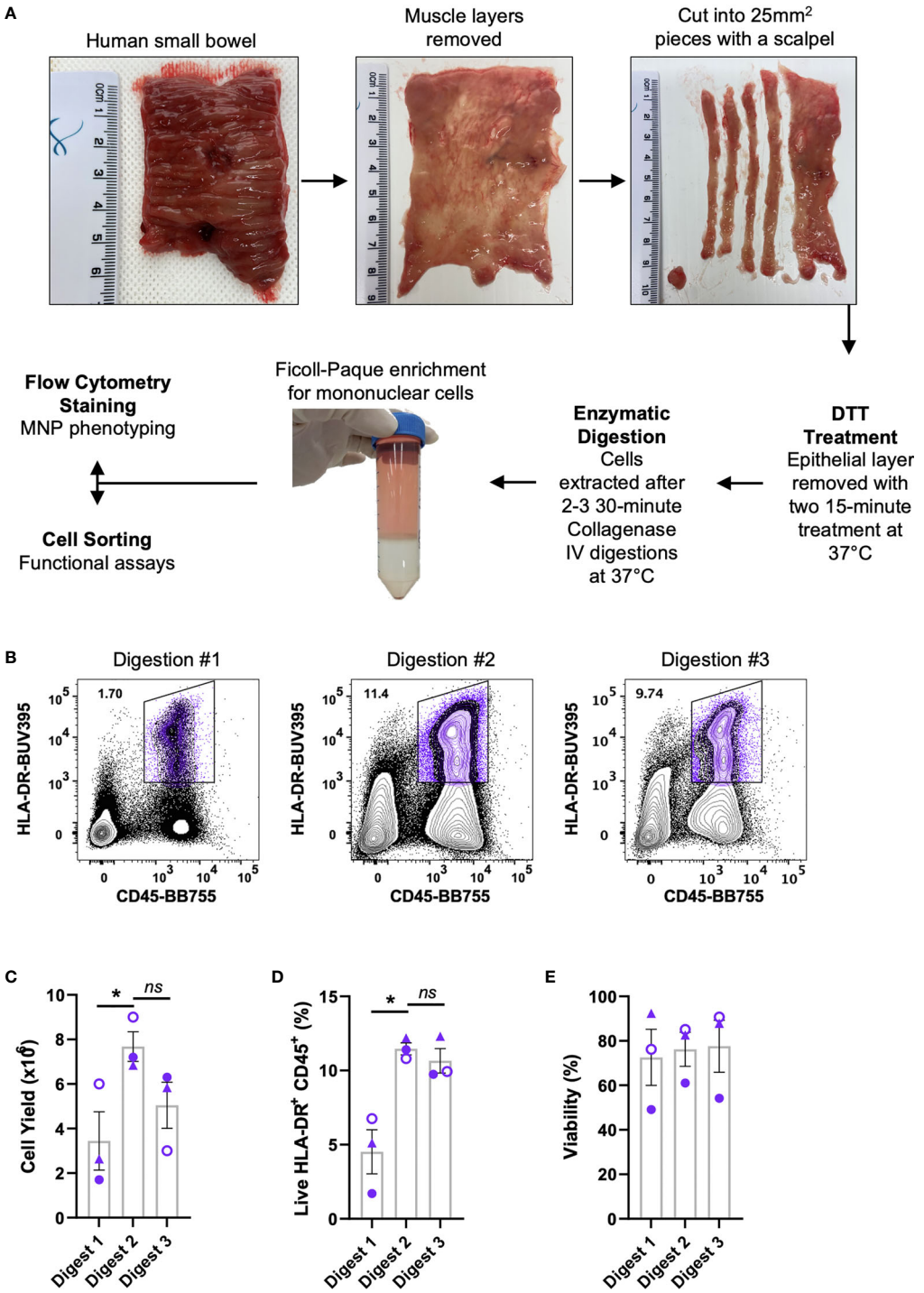

FIGURE 1 | Isolation of immune cells from human intestinal tissues. Discarded human intestinal tissues were obtained within one hour of surgery. **(A)** Underlying tissue was removed using curved surgical scissors before being diced into small pieces with a scalpel. Tissue was treated with DTT for two 15 min incubations at 37°C prior to 2-3 30 min digestions with collagenase type IV. Mononuclear cells were enriched using a Ficoll-Paque gradient before cells were stained for phenotyping or cell sorting. **(B)** Representative plot of live HLA-DR$^+$ CD45$^+$ proportions from each digestion. **(C)** Cell yields from each digestion, counted post-Ficoll on a haemocytometer. **(D)** Percentage of live HLA-DR$^+$ CD45$^+$ cells from total cells as determined by flow cytometry. **(E)** Percentage of viability of each digestion as determined by flow cytometry with mean ± SEM. Each symbol represents an individual donor (n=3). Statistics was by a Kruskal Wallis test with Dunn's multiple comparisons test comparing the sequential digests (*$p < 0.05$, ns, not significant).

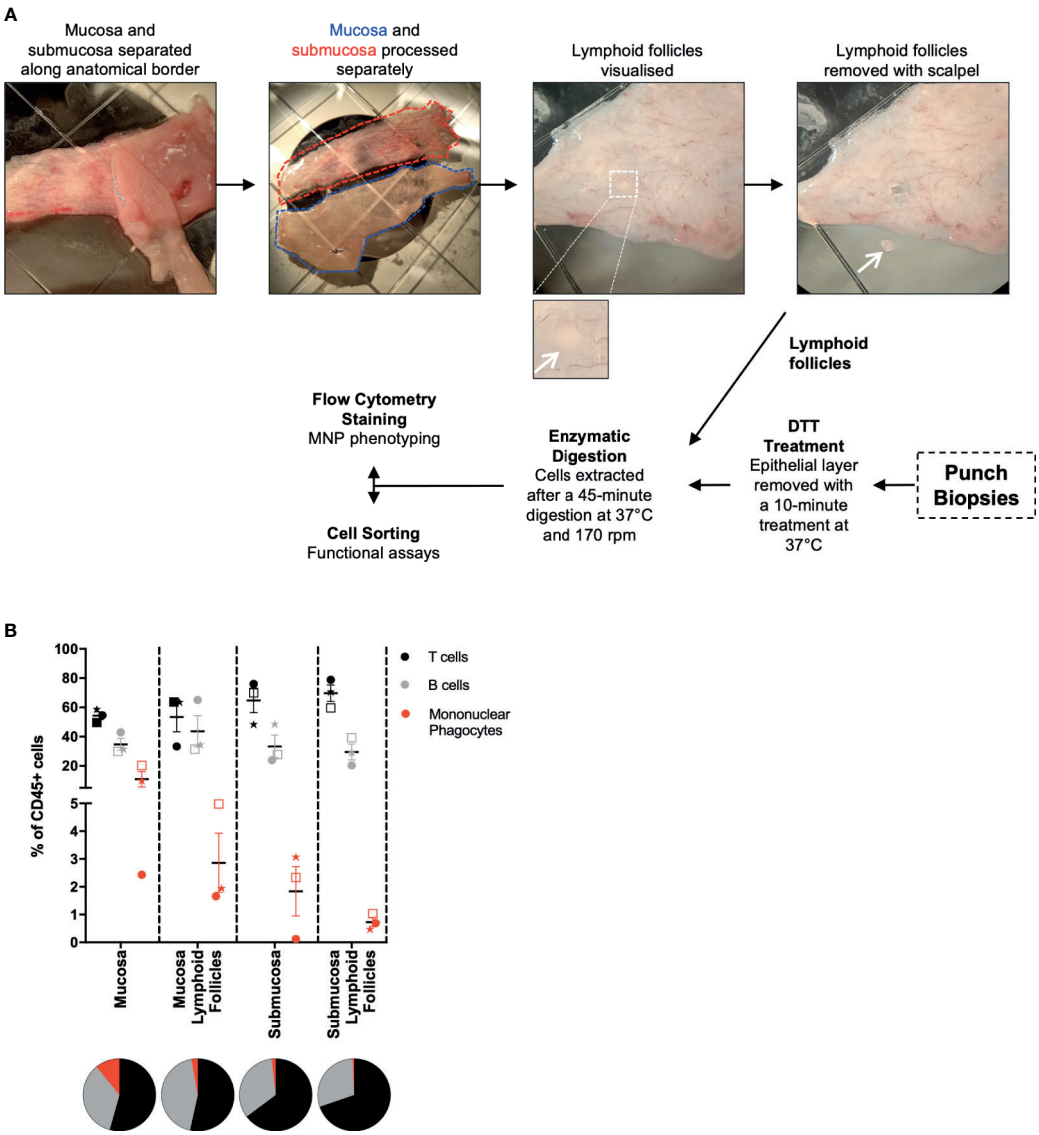

FIGURE 2 | Separation of tissue compartments from intestinal tissue. Discarded human intestinal tissues were obtained and underlying tissue removed as in **Figure 1**. **(A)** Under a dissecting microscope, the mucosa and submucosa were mechanically separated with forceps. Mucosa and submucosa were then processed separately for the removal of lymphoid follicles. Insert: magnified view of a lymphoid follicle. Follicles are visualised and removed from each tissue layer using a scalpel. Mucosa and submucosa were processed as per **Figure 1**, with no DTT treatment for the submucosa. Lymphoid follicles were digested for 45 mins at 37°C at 170 rpm. When obtained, punch biopsies were DTT-treated for 10 mins at 37°C, before being digested as per the lymphoid follicles. **(B)** Cells liberated from tissue compartments were analysed by flow cytometry. $CD3^+$ T cells, $CD19^+$ B cells and $HLA\text{-}DR^+$ mononuclear phagocytes were represented as a percentage of $CD45^+$ cells with mean ± SEM. Each symbol represents an individual donor (n=3). Pie charts represent the mean as parts of whole.

We confirmed that FCεR1α was expressed on some cDC2s using the clone AER-37, though it was not until the same cells were stained with the clone 9E1 that we could confirm that the epitope recognised by AER-37 was being partially cleaved by collagenase type IV (**Figure 3A**). In addition to epitope cleavage, a further consideration is whether markers found on the surface of circulating cells correspond to tissue DCs. Blood cDC1s have been classified as $CD141^+$ and $XCR1^+$ (19), however, this does not appear to be the case in intestinal tissues where only a small portion of $XCR1^+$ cells express CD141, the expression of which does not correlate to XCR1 expression (**Figure 3B**). Therefore, CD141 is not a reliable marker of cDC1s in the intestine. Taken together, these results emphasise the importance of marker and antibody clone selection for the proper identification of enzyme-liberated intestinal DCs.

Autofluorescent Correction of Tissue-Resident Macrophage Subsets *via* Flow Cytometry

Tissue-resident yolk sac derived macrophages are characteristically autofluorescent (AF) (20). This unique characteristic allows for their

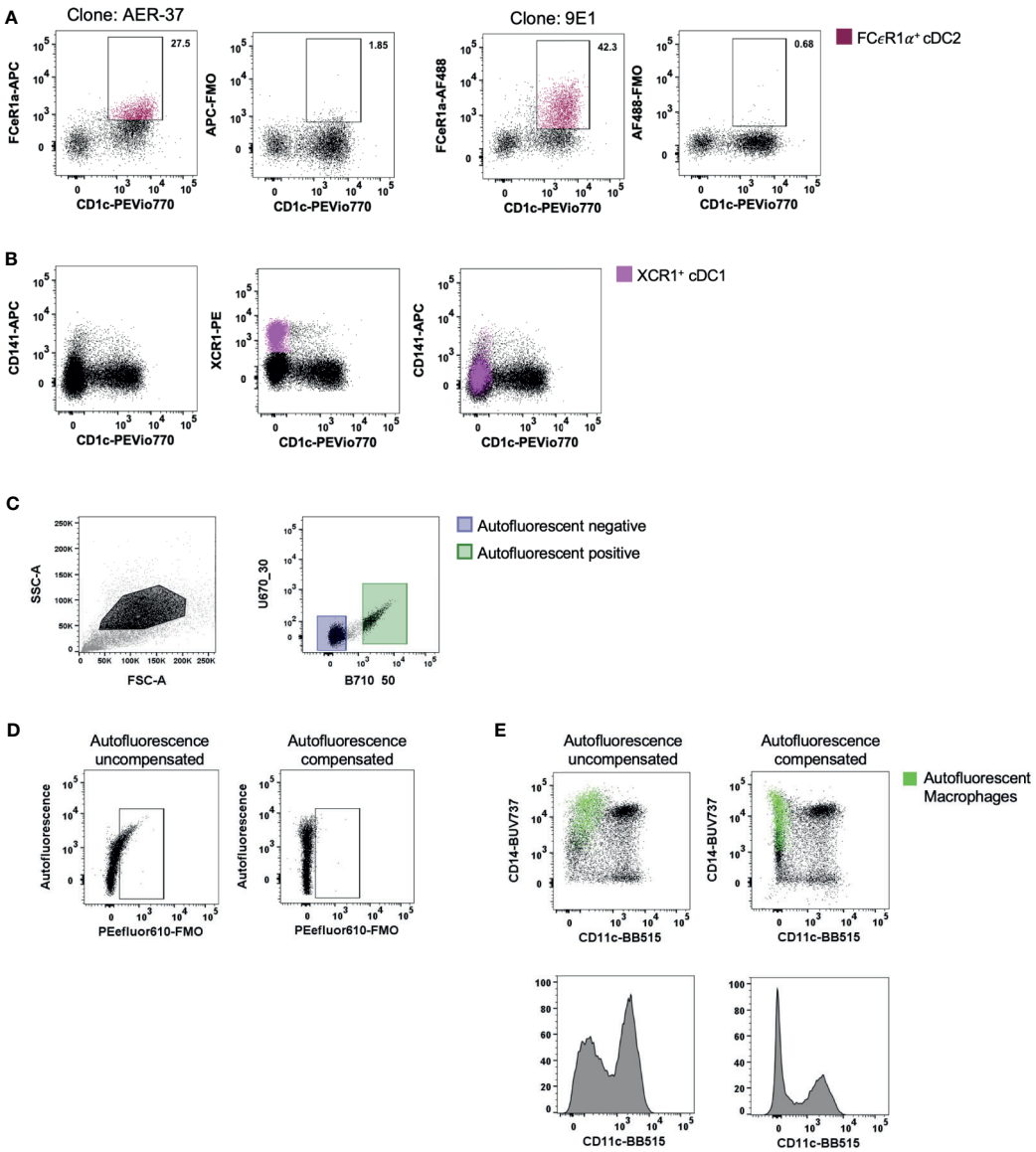

FIGURE 3 | Optimisation of staining and acquisition parameters for flow cytometry. Intestinal mononuclear phagocytes were isolated as per **Figure 1**. **(A, B)** Cells were stained to isolate HLA-DR$^+$ CD45$^+$ CD3$^-$ CD19$^-$ CD11c$^+$ DCs. **(A)** In the same donor, FCεR1α expression was compared between clones AER-37 and 9E1 for optimal expression, with corresponding Fluorescence Minus One (FMO) control. **(B)** Expression of defining conventional DC (cDC) 1 markers XCR1 and CD141 were compared in the same donor for correlation in intestinal tissue. **(C)** Representative plot of unstained intestinal cells, gated on events with higher side scatter. Autofluorescent negative and positive cells were gated and applied to un unused detector channel in FlowJo V10.7.1 to create the autofluorescent-corrected compensation matrix. **(D)** Representative plots for autofluorescent correction, showing correction of fluorescent signal for PEefluor610. **(E)** Representative plots of live HLA-DR$^+$ CD45$^+$ CD3$^-$ CD19$^-$ mononuclear phagocytes, comparing the CD11c BB515 signal with and without correction of autofluorescent signal. Autofluorescent macrophages overlaid, gated for CD14+ and autofluorescence.

identification by flow cytometry as AF CD14$^+$ (13, 20). However, as more subsets of macrophages are defined in the intestine (10), their AF properties can mask the expression of key defining markers as well as presenting false positives. Roca, Burton (21) recently described a method for AF correction using AutoSpill, an algorithm for calculating spillover coefficients, by assigning AF as an additional endogenous dye using an unstained sample. We acquired an unstained sample of intestinal-liberated cells in addition to single-colour controls on beads to facilitate AF correction for manual compensation. Using the compensation wizard in FlowJo, the unstained sample was allocated to an unused detector channel where autofluorescence spills naturally, for example B710_50 on the FACSymphony, treating the AF signal from tissue cells as a single-colour control. By setting positive and negative gates for AF (**Figure 3C**) and re-calculating the compensation matrix with AF as a measurable parameter,

AF spillover into other detectors was minimised, as shown with the correction of false signal using Fluorescence Minus One (FMO) (**Figure 3D**) and on $CD45^+$ $HLA\text{-}DR^+$ $CD3^-$ $CD19^-$ cells (**Figure 3E**). Furthermore, not using this method leads to inaccurate determination of surface marker expression as indicated for key DC expression marker CD11c in **Figure 3E**. Here we show that the $CD14^+$ $CD11c^-$ AF macrophages can be corrected to display as $CD11c^-$, instead of spilling false signal into the detector allocated to CD11c.

Identification of Intestinal-Derived Mononuclear Phagocytes

MNPs share several common markers in tissue making their definitive identification and characterisation challenging (8, 10). Using flow cytometry, we were able to identify collagenase-liberated MNPs ($CD45^+$ $HLA\text{-}DR^+$ $CD3^-$ $CD19^-$) in intestinal tissue. We identified four subsets of $CD14^-$ DCs: cDC1s expressing XCR1; two subsets of $CD1c^+$ cDC2 divided by langerin expression; and $CD123^+$ plasmacytoid DCs (pDCs) which, as expected, were more readily detectable in inflamed tissue (22) (**Figure 4A**). DC3s were identified as $CD11c^+$ $CD1c^+$ $CD163^+$ $CD88^-$ and $CD14^{+/-}$ (14, 15). We also characterised intestinal DCs by their expression of CD103 and SIRPα (11, 12). The $SIRP\alpha^+$ DCs correlate with the $langerin^+$ cDC2s, $CD103^-$ DCs correlate to $langerin^-$ cDC2s, and the $SIRP\alpha^-$ $CD103^+$ cells correlate with the $XCR1^+$ cDC1s (**Figure 4B**), showing that while there are distinct differences between human tissue DC phenotypes, there are overlapping similarities. In addition, $CD14^+$ macrophages could be divided into four subsets: Mf1 ($HLA\text{-}DR^{low}$), Mf2 ($CD11c^+$), Mf3 ($CD11c^-$) and Mf4 ($CD11c^-$ $CD11b^+$) (10), with Mf3 macrophages representing the largest proportion of mononuclear phagocytes (**Figure 4C**). We identified the Mf4 population in human jejunum (10), however have consistently been unable to identify these cells in lower intestinal tissues. Also identified were $CD14^+$ $CD88^+$ monocytes which can be further separated from macrophages by their expression of calprotectin (18). As whole tissue samples are not always abundantly available, we optimised a FACSort panel that would allow for the identification and isolation of MNPs from terminal ileum biopsies. As AF correction occurs post-acquisition, a gating strategy was devised to utilise AF spillover. We identified $XCR1^+$ cDC1, $langerin^{+/-}$ cDC2, pDCs as well as AF macrophages (Mf3) and $CD11c^+$ macrophages (Mf1 and Mf2) for use in functional assays (**Figure 4D**).

Mononuclear Phagocytes Are Phenotypically Immature at the Time of Liberation

A complication of the digestion process can be the inadvertent activation or maturation of cells (9, 23, 24). We investigated the maturation status of MNPs isolated by our digestion protocol, by assessing the surface expression of adhesion (CD54) and co-stimulatory molecules (CD80, CD83 and CD86), which are upregulated upon maturation (25). Immediately after isolation, all MNP subsets expressed low levels of CD54, CD80, CD83 and CD86 (**Figure 5A**). In two of three donors, Mf1s, Mf3s and cDC1s did not survive the 14-hour culture and therefore their maturation status at this time was not determined. However, significant upregulation of CD54 was seen on Mf2s, CD54, CD83 and CD86 on $langerin^{+/-}$ cDC2s, with $langerin^-$ cDC2 also upregulating CD80 post-culture (n=3) (**Figures 5B, C**). This culture-induced upregulation of maturation markers suggests that not only were the tissue-liberated MNPs viable, but they also remained immature throughout the enzymatic digestion.

Liberated Intestinal Mononuclear Phagocytes Can Be Used for Functional Assays *Ex Vivo*

Having isolated and identified immature MNPs, we next confirmed that their biological functionality had been maintained. Sorted MNPs were stimulated and cultured for 16 hrs to determine their cytokine response. We observed, *via* intracellular staining, that AF macrophages, cDC1s and cDC2s could produce TNF, IL-23p40 and IL-6 in response to R848 (**Figure 6**). This cytokine response suggests that the intestinal-derived MNPs were liberated in a functional state.

DISCUSSION

The ability to interrogate immune cells from fresh human tissue is critical for advancing our understanding of the role these cells play in human disease settings. This is especially the case with intestinal MNPs, which are integral components of the mucosal innate immune system. Here, they play a crucial role in maintaining tolerance of the commensal microbiota while also remaining poised to respond to invading pathogens. While sophisticated animal models have greatly expanded our knowledge of intestinal MNPs, they are not able to completely recapitulate human tissue with many cells differing phenotypically and functionally (12, 26). Translating the data generated from these animal models requires the development of isolation methods to interrogate human immune cells *ex vivo* while maintaining their biological state. We previously described tissue digestion protocols for the isolation of MNPs from human abdominal skin (9) and anogenital tissues (8, 13). However, given the distinct phenotypic, functional and structural differences between skin and mucosal tissue, we present an isolation protocol to extract functionally immature MNPs from human intestinal tissue. We have carefully selected the optimal antibody clones and corrected AF spillover to develop high-parameter flow cytometry gating strategies to accurately identify all currently known subsets of MNPs in fresh human intestinal tissue.

An important consideration when isolating cells *via* enzymatic digestion is the delicate balance between cell yield and viability. Many groups perform one round of enzymatic digestion (10, 11), however, Lefrançois and Lycke (27) showed the advantages of performing a second round of digestion in murine mucosal tissues to enhance cell yield and viability. Our findings show that we can digest human intestinal tissue for a total of 90 minutes without compromising cell viability or proportions. As a result, we can liberate a higher quantity of

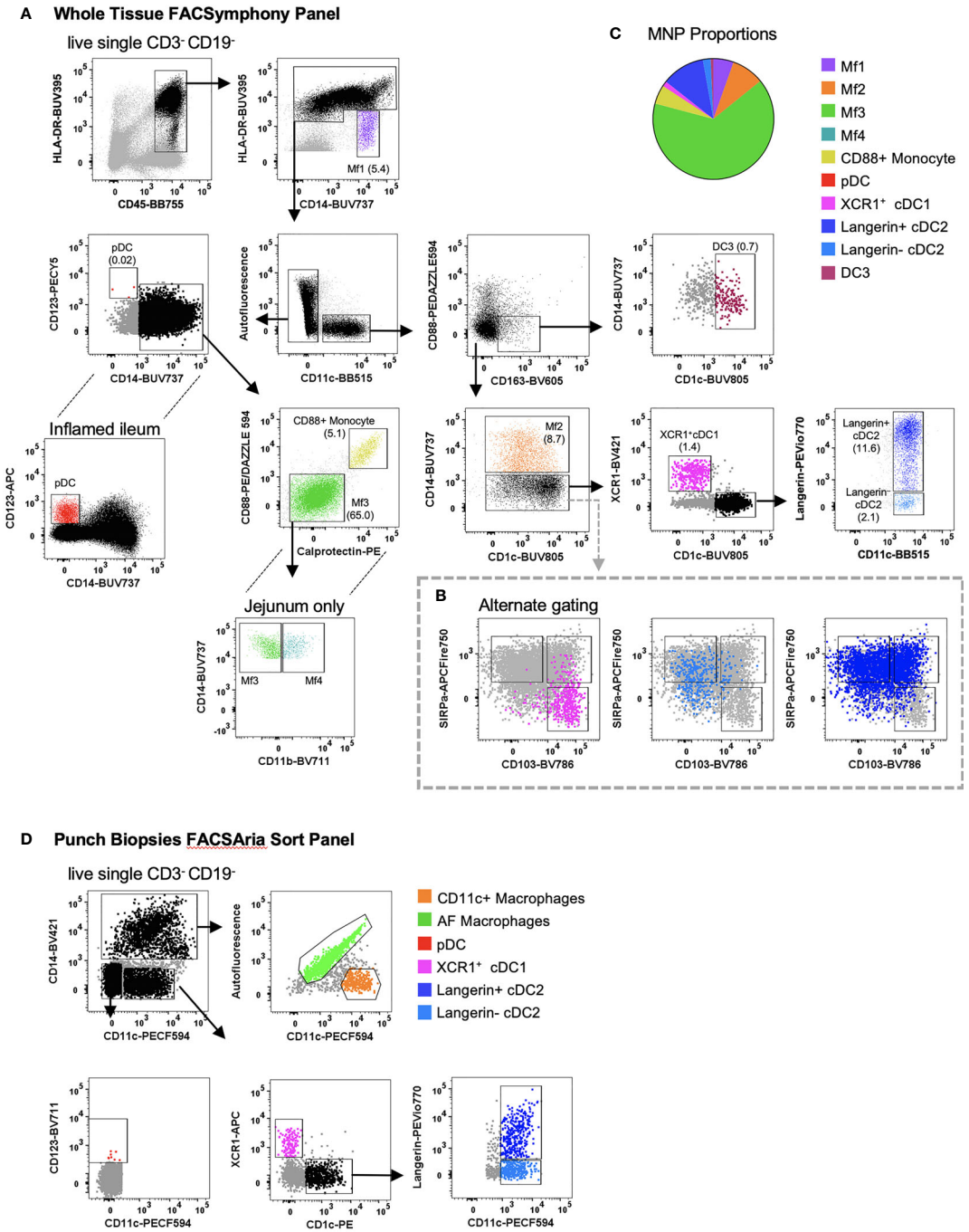

FIGURE 4 | Identification of intestinal-derived mononuclear phagocytes by flow cytometry. Intestinal mononuclear phagocytes were isolated as per **Figure 1**. **(A)** Cells were stained with FACSymphony Phenotyping Panel (**Table 1**). All mononuclear phagocytes were gated within the live, single, $CD45^+$ $CD3^-$ $CD19^-$ population. Intestinal mononuclear phagocytes (MNP) were gated in sequential order with percentage of total mononuclear phagocytes in brackets. Macrophage (Mf) 1 were defined as $CD14^+$ $HLA\text{-}DR^{low}$, with all sequential MNPs gated as $HLA\text{-}DR^+$. Cells were then divided by their expression of CD11c. $CD11c^-$ cells included $CD14^-$ $CD123^+$ plasmacytoid DCs (pDCs), $CD14^+$ $Calprotectin^+$ $CD88^+$ monocytes and $CD14^+$ $autofluorescent^+$ Mf3s which could be further subdivided into $CD11b^+$ Mf4 detectable only in the jejunum. $CD11c^+$ cells were divided as follows: $CD88^-$ $CD163^+$ $CD1c^+$ DC3s, $CD14^+$ Mf2s, $CD14^-$ $CD1c^-$ $XCR1^+$cDC1s, $CD14^-$ $XCR1^-$ $CD1c^+$ cDC2s divided by their expression of langerin. **(B)** Alternative gating for $CD11c^+$ $CD14^-$ cells, characterised by SIRPα and CD103, overlaid with cDC1 and cDC2s from the main gating strategy for comparison. **(C)** Pie cart representation of proportion of mononuclear phagocyte subsets as part of whole of all mononuclear phagocytes **(D)** Cells isolated from intestinal biopsies were positively selected for CD45 and stained with FACS Sort Panel (**Table 2**). Live, single $CD45^+$ $HLADR^+$ cells were divided by CD14 expression. $CD14^+$ cells were sorted as $CD11c^-$ $autofluorescent^+$ macrophages and $CD11c^+$ macrophages. $CD14^-$ cells were sorted as $CD123^+$ plasmacytoid DCs, $XCR1^+$ cDC1s, $CD1c^+$ cDC2s $langerin^{+/-}$.

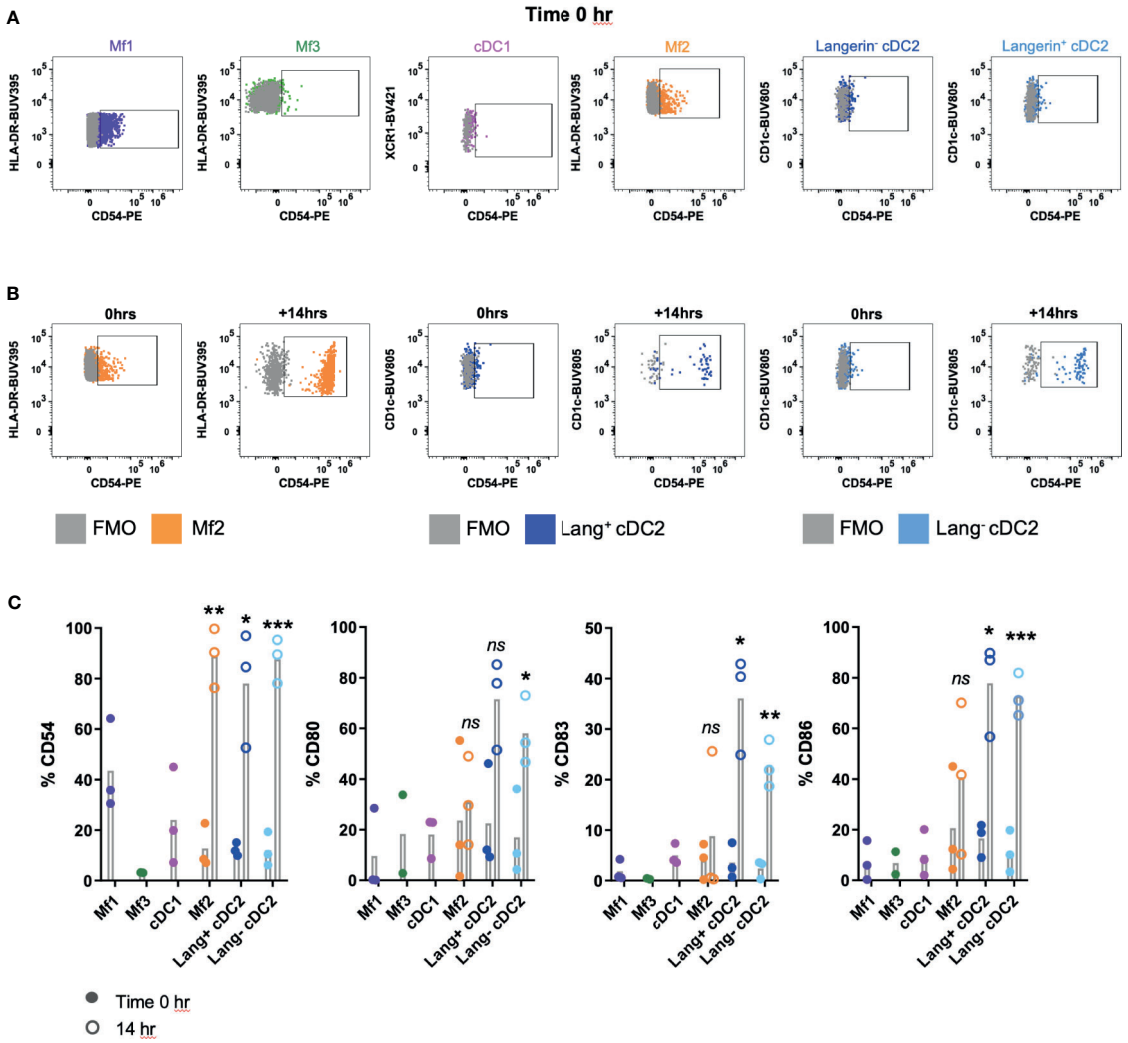

FIGURE 5 | Investigating the maturation phenotype of tissue MNPs liberated by enzymatic digestion. Intestinal mononuclear phagocytes (MNP) were isolated as per **Figure 1**. Cells were stained for flow cytometry with FACSymphony Phenotyping Panel (**Table 1**) with drop-ins for CD54, CD80, CD83 and CD86, immediately following liberation (time 0 hr) as well as 14 hr post-liberation. **(A)** Cells were gated as per **Figure 4A**, with representative plots of time 0 hr expression of CD54 shown, compared to Fluorescence Minus One (FMO). **(B)** Representative plot for Mf2, langerin$^+$ cDC2 and langerin$^-$ cDC2 showing expression of CD54 at time 0 hr compared to 14 hr post-liberation, compared to FMO. **(C)** Expression of CD54, CD80, CD83 and CD86 on MNP subsets at time of liberation (closed circles), with 14-hr comparison (open circles) for Mf2, langerin$^+$ cDC2 and langerin$^-$ cDC2 (n=3). Statistics was by a paired Student's t-test comparing each marker for each cell type at 0h with 14h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant).

total cells for downstream functional assays, which can be a limiting factor when working with human tissue-derived cells (13, 28). Most of the data we have presented here has taken advantage of our privileged access to whole pieces of intestinal tissue. While these larger tissue samples are often required to perform sorting for functional assays *ex vivo*, it is far more common to receive surgical biopsies from patients. To that end, we have shown that using our protocols it is possible to liberate, phenotype and even sort MNPs from human punch biopsies. Due to punch biopsies being smaller, a shorter digestion protocol is sufficient to liberate the cells which will make the interrogation of DC and macrophages from the intestine easier and far more accessible.

In the intestine, there are several different anatomical compartments containing varying ratios of functionally specialised immune cells each with their own unique interactions with the local intestinal environment (29). To interrogate these immunological compartments and the unique role each plays in disease settings it is crucial to isolate and interrogate them separately. The four primary compartments investigated in this study are the mucosal and submucosal layers, and their associated lymphoid follicles. Posing the greatest obstacle to deepening our knowledge of these immune compartments has been the difficultly in delicately separating the tissue layers and correctly identifying and removing the small lymphoid follicles. Other groups have attempted to expedite this task by using dyes to better visualise the lymphoid follicles (16),

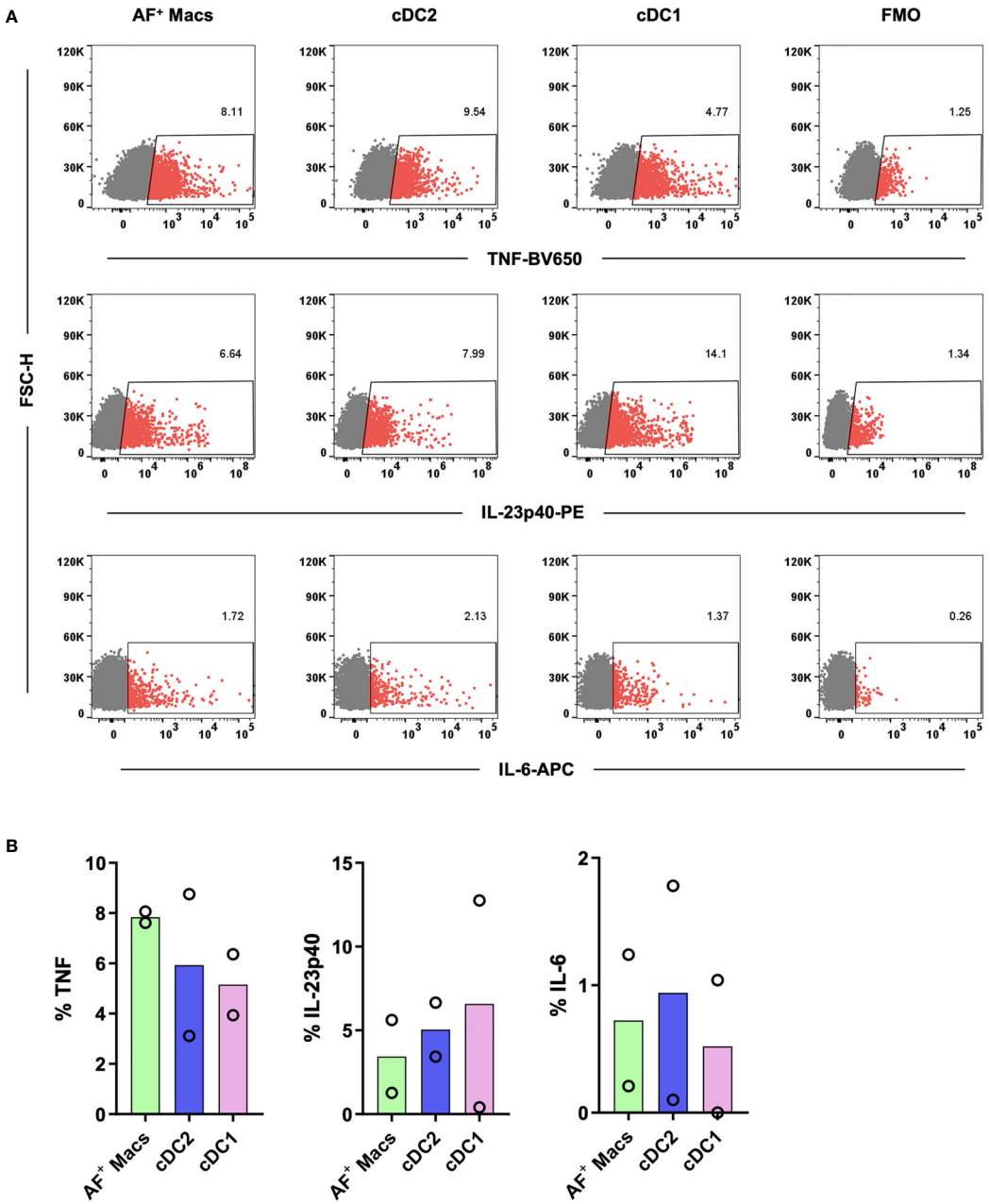

FIGURE 6 | Investigation of the cytokine responses of tissue MNPs liberated by enzymatic digestion. Intestinal mononuclear phagocytes were isolated as per **Figure 1**. Cells were positively selected for CD45 and stained with FACS Sort Panel (**Table 2**). Autofluorescent (AF) macrophages (Macs), conventional DC (cDC) 1 and cDC2s were sorted based on gating in **Figure 4C**. Sorted cells were cultured at 1x10^6 cells/mL in DC Culture Media with 1μg/mL R848 for 2 hours. 2.5μg/mL Brefeldin A was added, and the cells cultured for a further 14 hours. Cells were then stained with Fixable Viability Stain 700 and intracellularly stained with anti-TNF, anti-IL-23p40 and anti-IL-6. Cells were acquired on a FACSymphony. **(A)** Representative plots for expression of TNF, IL-23p40 and IL-6 on all subsets compared to Fluorescence Minus One (FMO). **(B)** Expression of TNF, IL-23p40 and IL-6 as a percentage of live single cells minus FMO, columns represent mean expression (n=2).

however, here we present an alternative method that removes this step and the risk it may pose in altering their function (30). Based on the method of Fenton, Jørgensen (16, 31), we were able to separate these compartments and show their unique immune profiles.

A known complication of enzymatic digestion is the cleavage of cell surface proteins, which can lead to the misidentification of cell subsets. We have previously shown that type IV collagenase best preserved the cell surface proteins used to identify tissue MNPs which led to the discovery of previously unidentified epidermal DC subset (8, 9). However, even with this collagenase blend, surface proteins can be partially cleaved, leading to underrepresentation of cell subsets within the tissue or their

loss of function. It has proven particularly challenging to identify tissue-specific cDC2s as their markers overlap with monocytes and macrophages. Dutertre, Becht (18) revealed that FCϵR1α was one of two exclusive markers used to identify tissue cDC2s. Upon reviewing the literature, we selected FCϵR1α clone AER-37 as this was the most widely used clone. By using this specific antibody clone and type IV enzyme combination, we observed that FCϵR1α was partially sensitive to enzymatic cleavage as an unusually low proportion of cDC2s were FCϵR1α^+. It wasn't until we selected a new FCϵR1α antibody clone: 9E1, that we were able to detect a much higher proportion of FCϵR1α expression on cDC2s in the intestine. Hence, FCϵR1α clone 9E1 with collagenase type IV is the superior combination.

Another consideration is the change in the pattern of marker expression on MNPs from different tissue compartments. In human blood and cutaneous abdominal tissue, cDC1s have been shown to express both CD141 and XCR1 (32). However, in human intestinal tissue we found that the expression of CD141 on cDC1s is extremely low and does not correlate with expression of XCR1. In addition, tissue cDC2s can upregulate their expression of CD141 (33). Peripheral blood cDC1s are defined by their CD141 and CADM1 expression, although CADM1 is not expressed by human skin cDC1s (9). We show here that CD141 is an unreliable marker in human intestine, therefore, we recommend XCR1 as the optimal marker for identifying cDC1, with the S154046E clone, in our experience as the only reproducibly reliable antibody that currently works on tissue-derived cells. This highlights the importance of carefully selecting antibodies that not only target preserved receptor epitopes post-digestion but reflect the expression of cell surface receptors in the tissue of interest.

The classification of MNPs is a rapidly evolving field of research and identifying them by flow cytometry can be challenging as subsets of MNPs share common markers (8, 10). This is further complicated by the AF properties of tissue-resident macrophages. Here we were able to correct AF by using an unstained tissue-derived control sample, allowing us to differentiate between real and false signal and improve resolution of surface markers used to identify MNPs. We could identify all currently known subsets of intestinal DCs using CD103 and SIRPα (11, 12) as well as the newly published DC3s (14, 15) in the same tissue. Furthermore, the SIRPα^- CD103$^+$ cells could be identified as XCR1$^+$ cDC1 and SIRPα^+ CD103$^{+/-}$ cells as CD1c$^+$ (langerin$^{+/-}$) cDC2, which is more relevant across different tissue types.

Following enzymatic extraction of MNPs from human intestinal tissue we have optimised methods to interrogate the function of these cells. This is important as extraction of MNPs from the tissue by enzymatic digestion can alter their biological function. Mature tissue MNPs have been shown to express elevated levels of leukocyte adhesion molecule CD54 (ICAM-1) and co-stimulatory molecules such as CD80, CD83 and CD86 (25). Conversely, immature MNPs freshly isolated from healthy human intestinal tissue should express low levels of these receptors on their surface. Following overnight culture, we showed that Mf2s and cDC2s had a substantial increase in maturation marker expression confirming that the cells were in an immature state upon liberation. Interestingly, we observed that Mf2s and cDC2s survived the culture, whereas Mf1s, Mf3s and cDC1 did not survive in two out of three donors. This suggests that cDC2 and Mf2, which are phenotypically very similar (10), share a propensity for survival outside of their tissue environment. We also showed that tissue liberated MNPs could produce cytokines in response to a TLR7/8 ligand. These findings confirm that intestinal MNPs liberated by enzymatic digestion following our optimised mucosal tissue digestion protocol are in an immature and biologically functional state. This is advantageous over other isolation methods such as spontaneous migration of DCs out of tissue during culture, which triggers the maturation of DCs limiting their use in functional assays (9).

In summary, our study demonstrates an enzymatic digestion protocol for the isolation of human intestinal MNPs in an immature state from tissue and biopsies while maintaining their biological function. We provide a method for the anatomical separation of the different gut-associated lymphoid tissues without the use of dyes. We also demonstrate the need for careful selection of antibodies that target preserved receptor epitopes and reflect their unique tissue-specific phenotype. These optimised protocols will greatly enhance our ability to interrogate APCs in human tissue, which will be important to understanding their role in homeostasis and diseases of the intestine.

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 Western Sydney Local Area Health District (WSLHD) Human Research Ethics Committee (HREC); reference number (4192) AU RED HREC/15 WMEAD/11. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CD and EV helped design and performed most of the experiments, analysed the data, prepared the figures and wrote the first draft of the manuscript. KB, HB and JR helped design, interpret and perform some of the experiments. MG, AD, GC, FR, JT, NP-N and GA provided the human tissue samples and intellectual input. GC and AC helped design the experiments and interpret the data. AH and SB supervised CD and EV, designed the experiments, analysed and interpreted the data, finalised the figures and wrote the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Targeting DCs for Tolerance Induction: Don't Lose Sight of the Neutrophils

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Chronic inflammatory disorders (CID), such as autoimmune diseases, are characterized by overactivation of the immune system and loss of immune tolerance. T helper 17 (Th17) cells are strongly associated with the pathogenesis of multiple CID, including psoriasis, rheumatoid arthritis, and inflammatory bowel disease. In line with the increasingly recognized contribution of innate immune cells to the modulation of dendritic cell (DC) function and DC-driven adaptive immune responses, we recently showed that neutrophils are required for DC-driven Th17 cell differentiation from human naive T cells. Consequently, recruitment of neutrophils to inflamed tissues and lymph nodes likely creates a highly inflammatory loop through the induction of Th17 cells that should be intercepted to attenuate disease progression. Tolerogenic therapy *via* DCs, the central orchestrators of the adaptive immune response, is a promising strategy for the treatment of CID. Tolerogenic DCs could restore immune tolerance by driving the development of regulatory T cells (Tregs) in the periphery. In this review, we discuss the effects of the tolerogenic adjuvants vitamin D3 (VD3), corticosteroids (CS), and retinoic acid (RA) on both DCs and neutrophils and their potential interplay. We briefly summarize how neutrophils shape DC-driven T-cell development in general. We propose that, for optimization of tolerogenic DC therapy for the treatment of CID, both DCs for tolerance induction and the neutrophil inflammatory loop should be targeted while preserving the potential Treg-enhancing effects of neutrophils.

Keywords: chronic inflammatory disorders, autoimmune disease, dendritic cell, neutrophil, tolerance, vitamin D3, corticosteroids, retinoic acid

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INTRODUCTION

A distorted immune balance can culminate in various chronic inflammatory disorders (CID) such as allergic asthma and autoimmune diseases, e.g., rheumatoid arthritis, systemic lupus erythematosus (SLE), and type 1 diabetes (T1D). CID are generally characterized by loss of tolerance for either self-antigens or harmless environmental antigens, resulting in the continuous production of inflammatory mediators, such as interferon-γ by T helper 1 (Th1) cells or interleukin-17 (IL-17) by Th17 cells (1). In allergic asthma, a Th2 cell response dominates with associated

cytokines IL-4, IL-5, and IL-13 (2). Generally, Th1 cells protect against intracellular pathogens like viruses and certain (myco) bacteria, whereas Th2 cells are indispensable for the eradication of helminthic pathogens (1, 2). Th17 cells are essential in the defense against fungi and bacteria, but they are pathogenic in the disease progression of multiple CID (1, 3, 4).

T-cell development is orchestrated by dendritic cells (DCs), specialized antigen-presenting cells, subsequent to the first-line response against pathogens by neutrophils, the major phagocytes of the innate immunity (5, 6). In recent years, compelling evidence has shifted our view of neutrophils from solely being short-lived first responders of the innate immune arm toward acting as accessory cells in adaptive immunity as well (6–8). Neutrophils promote the polarization of DC-driven T-cell development into Th17 cells *via* their granule content neutrophil elastase (NE) (9). DC-derived CXCL8 is processed into a short form by NE that promotes differentiation from human naive CD4 T cells to Th17 cells (9) (**Figure 1A**). In addition to their rapid recruitment to inflamed sites, neutrophils infiltrate draining lymph nodes *via* blood vessels, which was demonstrated in response to infectious agents, lysozyme immunization, or immune complexes in ovalbumin-immunized mice (10–15). At both sites, they are able to shape adaptive immunity by crosstalk with DCs and other immune or stromal cells, either by suppressing or by activating specific adaptive immune responses [reviewed in (6, 16, 17)].

In addition to IL-17, pro-inflammatory cytokine IL-22 and tumor necrosis factor alpha (TNF-α) are produced by Th17 cells. IL-22 induces the production of neutrophil-attracting chemokines by stromal cells, e.g., CXCL1 (18, 19). IL-17 and TNF induce the production of CXCL8 and granulocyte colony-stimulating factor (G-CSF) from epithelial cells, thereby increasing neutrophil activation and migration (20, 21) (**Figure 1B**). In turn, neutrophils chemoattract Th17 cells to the site of inflammation through the production of chemokines CCL2 and CCL20, ligands for the receptors CCR2 and CCR6, respectively, present on Th17 cells (22). Furthermore, neutrophils contribute to tissue damage and the overall inflammatory state in chronic diseases *via* the secretion of proteases and reactive oxygen species (ROS) and the formation of neutrophil extracellular traps (NETs) (23–25). NETs are composed of decondensed chromatin, histones, and granule proteins that serve as a useful tool to kill invading pathogens in host defense (6, 24). In rheumatoid arthritis and SLE, however, NET formation contributes to the disease activity as NETs are a source of autoantigens and they induce endothelial damage (26–28). Furthermore, NETs are released by neutrophils infiltrating the pancreas in T1D patients (25, 29). In the recent COVID-19 pandemic, NETs were also shown to contribute to disease severity (30, 31). Taken together, the recruitment of neutrophils to inflamed tissues and lymph nodes likely creates a highly inflammatory loop in CID through the induction of Th17 cells that should be intercepted to attenuate disease progression.

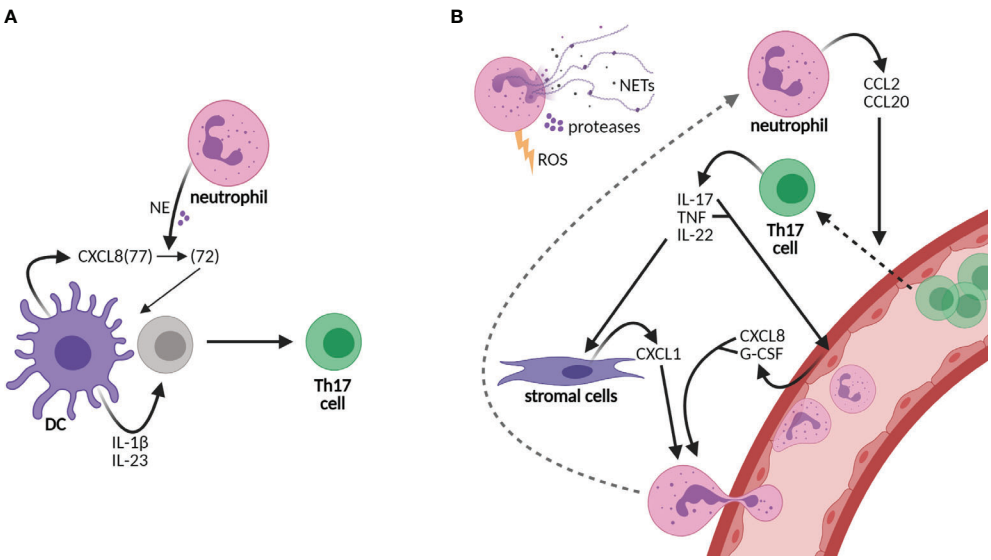

FIGURE 1 | Neutrophils sustain an inflammatory loop of Th17 cell development and recruitment to tissues. **(A)** Neutrophils shape the adaptive immunity by influencing dendritic cell (DC)-driven T-cell development, e.g., Th17 cell development by cutting DC-derived CXCL8(72) into the short form CXCL8(72), which is required for Th17 cell development from human naive CD4 T cells. Other cytokines required for Th17 cell development are IL-1β and IL-23. **(B)** Neutrophils are recruited to tissues *via* granulocyte colony-stimulating factor (G-CSF) and the chemokines CXCL1 and CXCL8, among others, of which production is promoted by Th17 cells. CXCL8 and G-CSF are produced by epithelial cells upon stimulation by IL-17 and/or tumor necrosis factor (TNF), while CXCL1 is released from stromal cells upon IL-22 stimulation. In turn, neutrophils produce CCL2 and CCL20, ligands for receptors CCR2 and CCR6, respectively, on Th17 cells, thereby elevating the infiltration of Th17 cells in tissues. Furthermore, neutrophils contribute to tissue damage *via* their release of neutrophil extracellular traps (NETs), proteases, and reactive oxygen species (ROS). Neutrophils contribute to both the development of Th17 cells in lymph nodes and the perpetuation of inflammation in tissues *via* the recruitment of Th17 cells.

A counterbalance to inflammatory Th cell activity is provided by regulatory T cells (Tregs), which develop in the periphery from naive precursors upon antigen presentation in the presence of specific tolerogenic factors, such as transforming growth factor beta (TGF-β) and IL-10 (32). Tregs can inhibit Th cell function by cell–cell contact or the secretion of inhibitory cytokines. A defect in either the number or the function of Tregs has been demonstrated in various autoimmune disorders (32). Immune tolerance could be restored *via* the induction of tolerogenic DCs that drive Treg development in the periphery (33, 34). Immunomodulatory agents such as vitamin D3 (VD3), corticosteroids (CS), and retinoic acid (RA) show potency to induce tolerogenic DCs (35–38). A treatment approach to inducing tolerogenic DCs should also take the additional role of neutrophils in steering DC-mediated T-cell development into account. In this review, we discuss the effects of the tolerogenic adjuvants VD3, CS, and RA on both DCs and neutrophils and their potential interplay.

DCs AS INDUCERS OF PERIPHERAL TOLERANCE

Although it is evident that DCs are paramount in the orchestration of the immune response toward a tolerogenic state, a dogma emerged that functionally immature DCs are the tolerogenic DCs, whereas mature DCs are always immunogenic DCs that elicit responses against pathogens (39). A key feature of mature DCs is their ability to migrate to lymph nodes where they activate naive T cells by presenting antigenic materials. While migratory DCs transport pathogen-derived antigens, they may also carry self-antigens and induce a non-inflammatory response. Therefore, mature DCs can be divided into tolerogenic or immunogenic DCs that are clearly distinguishable by the expressions of different sets of molecules, as reviewed by Lutz et al. (39). Tolerogenic DCs should rather be characterized by specific markers found on tolerogenic DCs and the different expression levels of molecules in comparison to immunogenic DCs, as indicated below.

Generally, the expressions of major histocompatibility complex II (MHCII) molecules and the activation markers CD80 and CD86 are reduced in tolerogenic DCs compared to immunogenic DCs. Tolerogenic DCs have been shown to induce T-cell anergy *in vitro* (39, 40). However, in the presence of TGF-β, FoxP3$^+$ Tregs are induced rather than anergic T cells (41, 42). Suppressed release of IL-12p40, a subunit of both IL-12 and IL-23, is required for the induction of Tregs, given that IL-12 alters the polarization of TGF-β cultured T cells from FoxP3$^+$ Tregs toward Th1 cells (39, 43, 44). Another CD4$^+$ Treg subset is that of Tr1 cells, characterized by a high expression of IL-10. The principal cytokine driving the generation of Tr1 cells is IL-10 (45). A specific human tolerogenic DC subset, termed DC-10, secretes high levels of IL-10, but no IL-12, and DC-10 potently induces Tr1 cells (46). TGF-β has no effect on Tr1 cell induction, while IFN-α, synergistically with IL-10, enhances Tr1 cell polarization (47). In addition to releasing TGF-β and IL-10, tolerogenic DCs express immunomodulatory molecules such as programmed death ligand 1 (PD-L1) and inducible co-stimulatory ligand (ICOSL), which induce Tr1 cells *via* their respective receptors, PD-1 and ICOS, on T cells (34, 39, 48–50). Another tolerogenic DC feature is the expression of the inhibitory receptor immunoglobulin-like transcript (ILT)-3, which has been associated with the increased generation of Tregs (46, 49, 51, 52). Furthermore, immunoregulatory enzymes can be upregulated by tolerogenic DCs, such as indoleamine-2,3-dioxygenase (IDO), which leads to a decreased T-cell proliferation and the induction of Tregs (53–55). Taken together, compared to immunogenic DCs, tolerogenic DCs are generally characterized by lower expressions of CD80/86, MHCII, and IL-12, while they secrete TGF-β and IL-10 and express tolerogenic markers such as PD-L1, ICOSL, ILT-3, and IDO.

NEUTROPHILS SHAPE DC-DRIVEN T-CELL DEVELOPMENT

In addition to the modulation of T-cell responses by neutrophils *via* secreted mediators or cell–cell contact, neutrophils shape the adaptive immune response *via* the modulation of DCs (**Figure 2**) (7, 8, 16, 56). The half-life of neutrophils was shown a decade ago to be 5 days in human circulation (57). Previously, it had been described that the life span of neutrophil, originally estimated at 8 h in circulation, could be prolonged in inflamed tissues *via* activating signals such as microbial products or cytokines (58). When neutrophils become activated to release granule contents, such as lactoferrin, these proteins can affect DCs and, consequentially, T-cell polarization, as reviewed in Breedveld et al. and in Minns et al. (8, 16) Lactoferrin was shown to induce DC maturation of immature human DCs through upregulation of the expressions of CD83, CD80/86, and human leukocyte antigen (HLA)-DR isotype (59, 60). Consistently, the T-cell stimulatory capacity of DCs is increased by lactoferrin treatment (60). Furthermore, neutrophil-derived ROS may increase DC maturation, given that hydrogen peroxide increased the expressions of CD86 and HLA-DR on immature human monocyte-derived DCs (moDCs) (61). Moreover, hydrogen peroxide suppressed the Treg-inducing capacity of murine DCs (62). Therefore, the potential effects of neutrophil-derived ROS on DCs and T-cell development should be investigated.

In addition to lactoferrin, other granule components such as cathelicidin (LL-37), NE, and myeloperoxidase (MPO) modulate adaptive immune responses *via* their effects on DCs (**Figure 2**). Similar to lactoferrin, cathelicidin induces DC maturation and enhances the secretion of Th1-inducing cytokines (63). NE is required for the development of Th17 cells in humans, as DC-derived CXCL8(77) is cleaved into a short form that promotes Th17 cell polarization (**Figure 1A**) (9). On the other hand, MPO suppresses DC activation and IL-12 cytokine production (8, 16). Supporting the potential anti-inflammatory effects of neutrophils on DC-driven T-cell development, NE was shown to induce the production of TGF-β in human DCs *in vitro*, which favored polarization toward FoxP3$^+$ Tregs (64, 65). Furthermore, NE impedes CD80/86 upregulation and the antigen-presenting

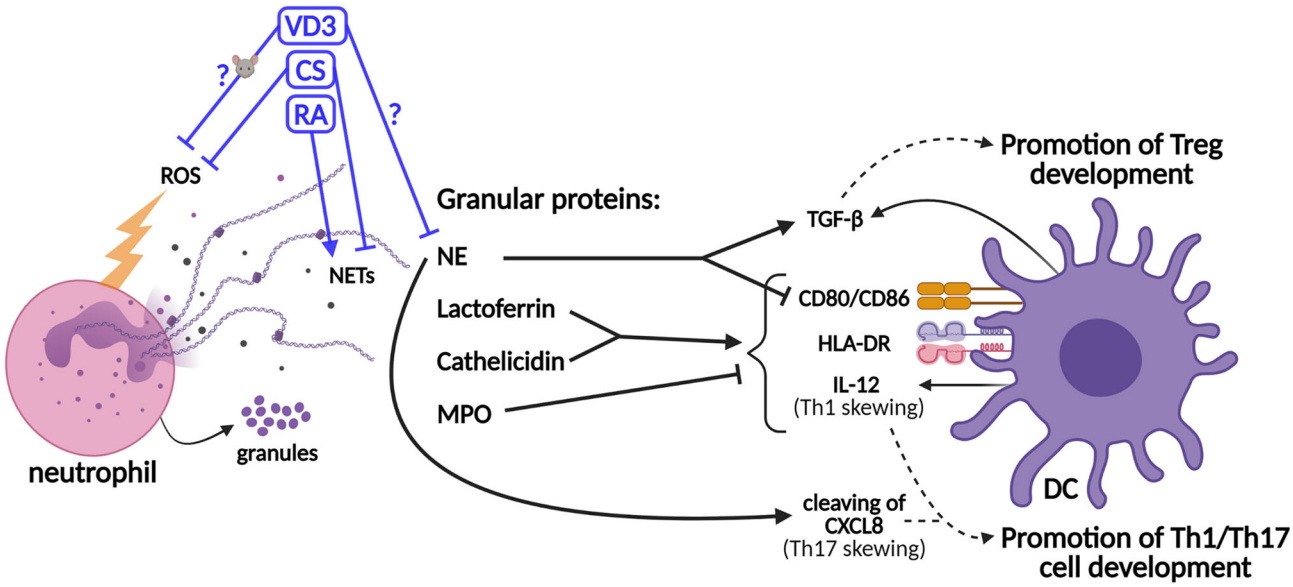

FIGURE 2 | Schematic representation of the effects of neutrophil granule contents on dendritic cell (DC)-driven T-cell development and the effects of tolerogenic adjuvants on the functions of neutrophils. Granule proteins released by neutrophils modulate T-cell development *via* direct effects on DCs. The production of the co-stimulatory molecules CD80/86, the major histocompatibility complex II (MHCII) molecule HLA-DR (human leukocyte antigen—DR isotype), and IL-12 cytokine is upregulated by lactoferrin and cathelicidin, while myeloperoxidase (MPO) inhibits this. Neutrophil elastase (NE) cleaves CXCL8, which promotes Th17 cell development. However, NE also stimulates the secretion of TGF-β by DCs and inhibits the expressions of CD80/86. TGF-β promotes the development of Tregs, while IL-12 and cleaved CXCL8 promote the development of Th1 and Th17 cells, respectively. The effects of vitamin D3 (VD3), corticosteroids (CS), and retinoic acid (RA) on the release of neutrophil extracellular traps (NETs) (or NE specifically) and the generation of reactive oxygen species (ROS) are shown. The effects of VD3 and RA on (human) neutrophils are unclear and experimental data are scarce.

ability of stimulated human moDCs (66). Hence, some granule contents were found to exert anti-inflammatory effects on DCs, thereby potentially even contributing to tolerance induction, while others stimulate DCs to facilitate Th1/Th17 cell development. Granule components decorate NETs to a different extent, as shown by Parackova et al. (67) The composition of NET differs substantially between pediatric T1D patients and healthy donors, with T1D NETs containing significantly more NE but less MPO and cathelicidin. T1D NETs induce significantly higher expressions of CD86 and HLA-DR on moDCs and elevate their production of the pro-inflammatory cytokines IL-6, CXCL8, and TNF when compared to healthy donor NETs (67). The relative abundance of granule proteins in NETs might alter the outcome of NET formation on adaptive T-cell responses, either promoting Th1/Th17 cell development or Treg development. Taken together, a delicate and intricate interplay between neutrophils (and their contents) and DCs orchestrates adaptive T-cell responses.

TOLEROGENIC ADJUVANTS: EFFECTS ON DCs AND NEUTROPHILS

The use of 1,25-dihydroxyvitamin D3, calcitriol, the active form of VD3, is one of the most widely established protocols for the generation of tolerogenic DCs (36, 68). VD3 activates intracellular metabolic pathways in DCs *via* the PI3K/Akt/mTOR pathway that regulates glycolysis, retaining DCs in a more immature state with reduced expressions of CD80/86 and HLA-DR (36, 69, 70). VD3 reduces the production of IL-12 in DCs through suppression of NF-κB activity (70–72). Additionally, VD3 enhances the production of IL-10 by DCs and thereby favors the development of IL-10-producing Tregs (49, 69, 73). The expressions of the inhibitory receptors ILT-3 and PD-L1 on DCs are induced by VD3 (48, 49, 52, 70). Furthermore, we have previously shown that the migration of $CD14^+$ dermal DCs, known for their tolerogenic effects, was increased by the intradermal application of VD3 in human skin explants (74). Dermal DCs primed with VD3 harbored less T-cell stimulatory capacity and altered T-cell polarization with increased Treg and reduced Th1 cell differentiation (74, 75).

Other well-recognized tolerogenic adjuvants are CS, which exert immunosuppressive effects *via* NFκB inhibition (36, 68, 76). Dexamethasone (Dex) is a commonly used synthetic CS. As shown for VD3, Dex reduces the expressions of CD80/86 on DCs and enhances their IL-10 production upon lipopolysaccharide (LPS) stimulation, while the release of IL-12 is suppressed. Correspondingly, Dex restrains the T-cell stimulatory capacity of DCs (48, 77). The tolerogenic DC features induced by Dex and VD3 largely overlap (36, 48) and may be complementary. Therefore, Dex and VD3 are also used simultaneously to

induce tolerogenic DCs (35, 68, 78), given that both adjuvants endow DCs with a wide range of tolerogenic properties.

In addition to vitamin D, the active metabolite of vitamin A, namely, RA, is a known tolerogenic adjuvant. $CD103^+$ DCs develop in response to RA, and these DCs promote tolerance to common harmless commensal bacteria in the gut (37, 79). RA-primed DCs induce the expression of the gut-homing receptor CCR9 on T cells, and they stimulate Tr1 cell development from naive T cells and $FoxP3^+$ Treg development in the presence of TGF-β (37). Furthermore, RA decreases the expressions of CD80/86 and HLA-DR on human moDCs and induces the production of IL-10 in DCs (38). Although RA has been described to induce tolerogenic DCs (37, 79), substantial debate is ongoing on the potential pro-inflammatory role of RA. During infection or tissue damage, RA is capable of inducing a pro-inflammatory DC phenotype, characterized by the release of IL-12 and IL-23 (80). An increased IL-12 release is at odds with the preconditioned suppressed release of IL-12 for the induction of Tregs (43, 44). Therefore, caution is warranted when considering RA as an adjuvant for tolerance induction *in vivo* given that RA potentially has pro-inflammatory effects, dependent on the inflammatory environment.

While the effects of these adjuvants on DCs are widely described, studies on the effects of VD3 on neutrophils are scarce and largely contradictory. Neutrophils were shown to express mRNA of the vitamin D receptor (81). Handono et al. showed that VD3 treatment of neutrophils from SLE patients inhibited the externalization of NE during phorbol 12-myristate 13-acetate (PMA)-induced NETosis, but the study is limited, with only five patients and no healthy control comparison (82). On the other hand, VD3 was suggested to play a pro-inflammatory role in facilitating the neutrophil defense against certain viruses since VD3 induced NETs and the expressions of Toll-like receptor 7 and IFN-α (83). Additionally, elevated production of the neutrophil chemokine CXCL8 by human neutrophils was reported with 1-day pretreatment with VD3 prior to LPS stimulation, while the LPS-induced IL-6 and TNF release was unaffected by VD3 (84). However, this was contradicted by others (81). Moreover, *in vitro* VD3 priming of murine neutrophils reduced their immune complex-induced ROS release, while in human neutrophils, VD3 did not suppress PMA-induced ROS generation (81, 85). Taken together, additional studies are required to determine the effects of VD3 on various neutrophil functions. If future studies support the observation that VD3 reduces the release of NE (82), VD3 could hypothetically reduce NE-facilitated Th17 cell development, thereby intercepting the neutrophil inflammatory loop (**Figure 1B**).

The effects of CS on neutrophils have been extensively studied (86). CS were shown to prevent neutrophil apoptosis, which enables neutrophils to exert their functions for an extended period (87). A well-established anti-inflammatory effect of CS on neutrophils is their inhibitory effect on the release of CXCL8 (88–90), thereby decreasing neutrophil recruitment that could intercept the neutrophil inflammatory loop and tissue damage (**Figure 1B**). Furthermore, CS attenuate other neutrophil functions such as L-selectin-dependent migration, ROS production, and NET formation (91–93). Despite these *in vitro* effects of CS on neutrophils, resistance to corticosteroid treatment is an ongoing problem in the treatment of neutrophil-associated asthma and chronic obstructive pulmonary disease. The reduced expression of the glucocorticoid receptor (GR) in airway neutrophils and an elevated ratio of the inactive isoform GRβ *versus* the active GRα in neutrophils could underlie this resistance (88, 89, 94). Overall, given the anti-inflammatory effects of CS on neutrophils, CS such as Dex seems to be a suitable candidate as a tolerogenic adjuvant for the treatment of CID. Hypothetically, reduced NET and the concomitant release of NE could restrict the development of Th17 cells, and the well-established inhibitory effect of CS on CXCL8 is beneficial for restrained neutrophil recruitment to tissue. However, the potential Treg-promoting effects of NE *via* enhanced TGF-β production by DCs, as shown by Maffia et al. (64, 65), should not be neglected (**Figure 2**).

A pro-inflammatory effect of RA on neutrophils was demonstrated in the limited number of studies that have investigated RA on neutrophil function. One report showed that a short RA pretreatment of isolated human neutrophils inhibited *N*-formyl-methionyl-leucyl-fenylalanine (fMLF)-induced ROS production (95), while in another study, a 4-h pretreatment with RA prior to fMLF stimulation increased the production of intracellular ROS (96). Additionally, RA was found to increase the NET formation of these neutrophils (96). Furthermore, a study in rats demonstrated that the functions of neutrophils, including ROS generation and chemotaxis, were reduced in rats fed with a RA-deficient diet, which were restored when supplemented with vitamin A (97). In conclusion, although data on the effects of RA on neutrophils are scarce, no evidence for anti-inflammatory effects exist, and most reports actually demonstrated that RA is required for neutrophil differentiation and for optimal neutrophil function (98).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The induction of peripheral tolerance in autoimmune diseases or other CID should be antigen-specific, given that broad immunosuppression can give rise to recurrent infections, for which treatment is problematic (99). Current treatment approaches using tolerogenic DCs for autoimmune diseases are based on the *ex vivo* generation of tolerogenic DCs, named tolDCs, by re-education of patient-derived DC progenitors into antigen-specific tolDCs using immunomodulatory agents such as VD3 or Dex (35, 36). Clinical phase I and II trials using tolDCs have been conducted for T1D and multiple sclerosis, and phase I trials in Crohn's disease and rheumatoid arthritis patients, as reviewed in Ten Brinke et al. (33) Due to the laborious and expensive nature of *ex vivo* tolDC generation, new approaches are in development for the *in vivo* induction of tolerogenic

programs in DCs. These new *in vivo* approaches are focusing on selective targeting of disease-relevant autoantigens toward (inhibitory) DC receptors, resulting in an antigen-specific anti-inflammatory response (100). Alternatively, nanoparticles or liposomes can be targeted to DCs (76, 101). These carriers can be loaded with self-antigens and tolerogenic adjuvants, as discussed above (76, 101, 102). The addition of VD3 to a peptide-loaded liposome enhanced the development of Tregs in mice and decreased the differentiation of antigen-specific Th1 and Th17 memory cells (103). These data suggest that the development of both pathogenic Th1 and Th17 cells could be diminished by *in vivo* tolDC therapy, while the development of Tregs is enhanced, which could greatly ameliorate the disease course in patients suffering from various autoimmune diseases (1).

Even though the aim of tolerogenic therapy *via* DCs is to specifically target DCs with nanoparticles loaded with antigens and adjuvants, off-target effects could occur and the encapsulated adjuvant could influence the functions of other cell types. We discussed the effects of the commonly used tolerogenic adjuvants on neutrophils and the potential desired outcomes in view of intercepting the neutrophil inflammatory loop (**Figure 1**). Additionally, reduced neutrophil recruitment to tissue and the anti-inflammatory effects of these adjuvants could dampen neutrophil-induced tissue damage, e.g., by NET release, which is often associated with the exacerbation of CID (23, 24, 26–28). CS show the most profound anti-inflammatory effects on neutrophils, followed by VD3, but this requires further investigation. The possibility that neutrophils contribute to the development of Tregs, for example *via* the production of TGF-β, NE-induced TGF-β release by DCs, or *via* neutrophil-derived apoptotic bodies, should also be studied (64, 65, 104, 105). This would indicate that an intricate balance of dampening the inflammatory effects of neutrophils, such as NE release that facilitates the development of Th17 cells, while preserving their potential Treg-promoting effects may be desired for CID treatment. Alternatively, if such an intricate balance cannot be achieved and the overall function of neutrophils is dampened by treatment, the Treg-promoting effects could solely be provided by tolerogenic DCs. Analysis of the number and function of Tregs in patients with neutrophilic disorders, such as in chronic granulomatous disease (CGD) patients characterized by defective ROS production or in congenital neutropenia patients with mutations in the NE gene (*ELANE*) (9, 106), could provide valuable insights into the effects of neutrophils on Tregs. In CGD patients, the number and function of $FoxP3^+$ Treg are not altered compared to that in healthy controls, while children with autoimmune neutropenia presented with a reduced frequency of $FoxP3^+$ Tregs (106, 107). In conclusion, for the optimization and further development of tolerogenic DC therapy for the treatment of autoimmune diseases and other CID, neutrophils and their potential double-edged sword effects on DC-driven T-cell polarization should certainly be taken into account.

AUTHOR CONTRIBUTIONS

FH performed the literature search, wrote the manuscript, and created all figures with BioRender.com. TGK and EdJ critically read and carefully revised all versions of the manuscript, providing valuable guidance and insight. All authors contributed to the article and approved the submitted version.

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The Therapeutic Potential of Tackling Tumor-Induced Dendritic Cell Dysfunction in Colorectal Cancer

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Colorectal cancer (CRC) is the third most diagnosed malignancy and the second leading cause of cancer-related deaths worldwide. Locally advanced and metastatic disease exhibit resistance to therapy and are prone to recurrence. Despite significant advances in standard of care and targeted (immuno)therapies, the treatment effects in metastatic CRC patients have been modest. Untreatable cancer metastasis accounts for poor prognosis and most CRC deaths. The generation of a strong immunosuppressive tumor microenvironment (TME) by CRC constitutes a major hurdle for tumor clearance by the immune system. Dendritic cells (DCs), often impaired in the TME, play a critical role in the initiation and amplification of anti-tumor immune responses. Evidence suggests that tumor-mediated DC dysfunction is decisive for tumor growth and metastasis initiation, as well as for the success of immunotherapies. Unravelling and understanding the complex crosstalk between CRC and DCs holds promise for identifying key mechanisms involved in tumor progression and spread that can be exploited for therapy. The main goal of this review is to provide an overview of the current knowledge on the impact of CRC-driven immunosuppression on DCs phenotype and functionality, and its significance for disease progression, patient prognosis, and treatment response. Moreover, present knowledge gaps will be highlighted as promising opportunities to further understand and therapeutically target DC dysfunction in CRC. Given the complexity and heterogeneity of CRC, future research will benefit from the use of patient-derived material and the development of *in vitro* organoid-based co-culture systems to model and study DCs within the CRC TME.

Keywords: metastatic colorectal cancer, cancer immunity, dendritic cell defects, immunotherapy, tumor microenvironment, immunosuppression, patient-derived organoids

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common and deadliest cancers worldwide (1, 2). At early stages of localized disease, surgical resection leads to a good prognosis or even cure (3). Unfortunately, more than half of the CRC patients develop metastasis, either at the time of diagnosis or later as relapse (4). The most common metastatic site is the liver, but metastasis can

also be found in the lungs, peritoneum, bones, and brain. Distant metastases are increasingly resected with curative intent (5, 6). For unresectable or recurrent metastatic disease, standard chemotherapies, as well as targeted treatments, have improved median overall survival to over 30 months (7–9). Despite these significant advances, metastatic CRC (mCRC) patients remain largely unresponsive to (immuno)therapy resulting in a 5-year survival rate of only 12% (1). At the moment, different therapeutic approaches are being actively investigated to fulfil the unmet need for therapies in mCRC (10, 11).

As with most cancers, CRC develops and spreads by evading immunosurveillance. To subvert the immune system, tumors evolved a number of escape mechanisms including loss of tumor antigens, upregulation of inhibitory molecules, and the generation of an immunosuppressive environment which recruits and corrupts stromal and immune cells (12). The CRC tumor microenvironment (TME) prevents immunosurveillance, supporting tumor growth and progression (13, 14). Besides hampering anti-tumor immunity, the generation of an immunosuppressive environment also hinders the success of immunotherapies (10, 14).

Dendritic cells (DCs), also known as professional antigen presenting cells, are a key immune cell type often impaired by the immunosuppressive TME. DCs are the central players in triggering, coordinating and amplifying anti-tumor immune responses, and in driving the clinical success of immunotherapies (15–17). However, in the presence of immunosuppressive signals, such as the ones released by tumor cells, DCs become dysfunctional and induce tolerance. Several studies suggest that tumor-mediated impairment of DC functions is decisive for immune evasion, tumor growth, metastasis initiation, and treatment resistance in different cancers including CRC (17–23). Despite the key role of functional DCs in anti-tumor immunity and treatment response, it is still largely unclear how CRC shapes DC fate.

The main aim of this review is to provide an overview of the current knowledge on how primary and metastatic CRC-driven immunosuppression affects DC phenotype and functionality. We will assess how this correlates with disease progression, mCRC patients' prognosis and treatment response. Moreover, the potential of therapies to revert DC defects in CRC will be discussed. From there, knowledge gaps will be pinpointed as unexplored avenues to study and target DC dysfunction in CRC.

COLORECTAL CANCER

CRC comprises a highly complex, heterogenous, and lethal group of diseases. Several factors contribute to CRC development and have implications in treatment response. As mentioned, the survival and treatment options of CRC patients largely depend on the stage, i.e., the extent of tumor invasion and spread at the time of diagnosis. Outgrowth of metastasis is facilitated by synchronous undetectable disseminated metastatic cells, tumor shedding into circulation, and therapy-induced immune impairment. As such, early detection of CRC through screening becomes a crucial factor to reduce mortality of the disease. However, disease stage is not always predictive for patient response and outcome. CRC patients at the same stage might have different disease progression based on the molecular heterogeneity of the tumor and the composition of the TME (24–27).

Tumorigenesis

CRC develops from a multistep accumulation of genetic and epigenetic alterations (28). The majority of CRC cases arise from sporadic mutations due to an interplay of environmental and lifestyle factors, the remaining due to genetic predisposition (29, 30). CRC develops from abnormal proliferation of mucosal epithelial cells of the large intestine, named polyps or adenomas, which can evolve to adenocarcinomas. CRC can develop from adenoma to carcinoma through one or a combination of different molecular mechanisms, namely chromosomal instability, CpG island methylation, and DNA mismatch-repair deficiency (24). Different sequential driver mutations associated with tumorigenesis occur in the APC/β-catenin, KRAS, MAPK and BMP/TGF-β pathways, as well as in tumor suppressor genes, such as TP53, at later stages (31, 32). As mutations accumulate, adenocarcinomas become invasive and spread to distant sites in the body establishing metastasis.

Depending on the underlying driving mechanism of genomic instability, CRC tumors can be broadly classified into (1) microsatellite instability-high (MSI-H), which account for ~15% of tumors and (2) microsatellite stable (MSS) accounting for the remaining ~85% of the cases (33). MSI tumors are characterized by high frequency of replication errors due to defective DNA mismatch repair mechanisms, which lead to a hypermutated state. Typically, MSI tumors are highly immunogenic, present high percentage of immune infiltrates, and are associated with a more favorable prognosis (34). In contrast, MSS tumors, which account for the large majority of CRC cases, are poorly immunogenic with low mutational burden, and are linked to poor prognosis (31, 32).

In mCRC, treatment with immune checkpoint inhibitors presents promising responses only in a minority of patients, with MSI tumors, while MSS tumors do not respond to PD-1 or PD-L1 inhibitors (35, 36). Unresponsiveness of MSS tumors to immune checkpoint inhibition and other immunotherapies has been associated with the low number of tumor-specific neoantigens, lack of infiltrating immune cells, and tumor-mediated immunosuppression (37). This implies that, to date, the vast majority of mCRC patients does not qualify for immunotherapy.

Besides MSS/MSI stratification, a more comprehensive classification system for CRC has been developed. The Consensus Molecular Subtype system, which divides CRC patients in 4 subtypes based on transcriptome analysis of the tumor and associated stromal and immune cells (38, 39). This stratification system suggests that characterizing gene expression of not only tumor cells but also of surrounding tumor-associated cells (such as fibroblasts, leukocytes and endothelial cells) allows better stratification of patients and confers higher predictive value for prognosis, management, and selection of appropriate

treatment (40). This emphasizes the importance of studying both the tumor and its surroundings.

The Tumor (Immune) Microenvironment

CRC initiation, progression, metastatic dissemination, and treatment resistance is not only driven by the accumulation of genomic and epigenomic aberrations but also by intricate and dynamic interactions between malignant and neighboring cells in the TME (41–44). Surrounding cells comprise endothelial cells, gut microbiota, cancer-associated fibroblasts (CAFs), and immune cells including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells, DCs, and T cells (45). Tumor cells are well-known for having a strong modulatory effect, for being able to recruit, corrupt or re-educate surrounding cells towards tumor-promoting phenotypes that foster tumor growth and spread.

Importantly, CRC generates a strong immunosuppressive TME that hampers immunosurveillance and allows immune evasion. To escape eradication by the immune system CRC recruits and polarizes immunosuppressive regulatory T cells (Tregs), TAMs, and MDSCs. In addition, CRC inhibits or excludes immune cells with anti-tumor potential such as DCs, NK cells, and effector T cells from the TME. By regulating local and systemic immune function, CRC creates immune impairments and an environment propitious for tumor growth and dissemination (46). Several studies have reported that CRC-induced local and systemic immune dysfunctions are closely associated with patient prognosis and sensitivity to therapy (47–51).

The intricate web of interactions within the TME is mediated by cell-to-cell contact and soluble factors, such as cytokines, chemokines, and growth factors, derived from the tumor and activated surrounding cells. These factors are constantly remodeling the TME and have not only local but also systemic effects, which are crucial for generalized immunosuppression enabling the generation of pre-metastatic niches and successful metastatic establishment (52).

One key immunosuppressive signaling molecule associated with CRC is transforming growth factor-β (TGF-β). High TGF-β expression in the TME of CRC has been linked to poor prognosis with a crucial role in successful tumor progression and metastasis development (40, 53–59). TGF-β modifies the TME by regulating infiltration and by suppressing or tweaking the phenotype of immune cells towards tolerance, impeding anti-tumor immunity (53, 60, 61). Consistent with these findings, neutralization of TGF-β signaling in the TME was found to impair liver metastasis establishment by unleashing T cell and NK cells anti-tumor responses, in different pre-clinical CRC models (14, 40, 54, 55, 58, 62). Promisingly, in a metastatic mouse model for CRC, treatment of established metastasis with anti-PD-L1 antibodies in combination with TGF-β blockade resulted in potent curative anti-tumor T cell-mediated immune responses (14). Besides TGF-β, other factors such as IL-6, IL-33, IL-8, IL-23, PGE2 and IDO-1 have shown similar immunomodulatory properties and impact in metastasis development and patient prognosis in CRC (63–74). These studies highlight the role and the potential of further exploring the interactions between malignant cells and immune cells, and combining current therapies with agents designed to target the TME (10, 13).

It is clear that regulation of immune cells by the immunosuppressive TME generated by CRC has a pivotal role in disease progression, not only by hampering immuno-surveillance but also by compromising the effectiveness of immunotherapies. Since, no effective treatment is available for the majority of mCRC patients, it becomes imperative to further understand how CRC interferes with immune activation for identification of new mechanisms for complementary therapies. DCs, as the main orchestrators of innate and adaptive anti-cancer immunity, appear as promising targets to unleash immune responses and immunotherapy efficacy.

DENDRITIC CELLS: THE HUB OF ANTI-TUMOR IMMUNITY

DCs comprise a heterogenous population of cells specialized in antigen capture, processing, and presentation. DCs act as hub of the immune system by initiating, linking and coordinating innate and adaptive immune responses (15, 16). DCs are key regulators of specific immune response owing to their unique capacity to (cross-)present antigens and prime T cells (15, 20). In anti-tumor immunity, DCs can promote T cell and NK cytotoxic activities, and also exert direct tumoricidal activity, sustaining cancer immunosurveillance. Consequently, DCs have been shown to have a crucial role in inhibiting local tumor growth, tumor dissemination, and metastatic establishment (17).

Tissue-resident DCs in steady-state conditions scan the environment for antigens and danger signals, acting as sentinels. In homeostatic conditions or under suppressive environmental cues, DCs present an immature and tolerogenic phenotype, characterized by low expression of co-stimulatory molecules and pro-inflammatory cytokines, inability to prime T cells, and secretion of immunosuppressive cytokines (e.g., IL-10 and TGFβ). This functional state ensures immune (self-) tolerance, through various mechanisms including T cell depletion and anergy, as well as generation of Tregs (15, 75–77).

If uptake and processing of (tumor) antigens occurs in the presence of danger signals and inflammatory cytokines, DCs undergo maturation (78). The maturation process encompasses several morphological, functional, and phenotypical changes, which include enhanced migration abilities through CCR7 expression, upregulation of co-stimulatory molecules CD80, CD83, and CD86, and secretion of pro-inflammatory cytokines such as IL-12, IL-6, TNF-α and IL1-β. All these signals, together with antigen presentation on major histocompatibility complexes (MHC), are required for proper priming, activation, and proliferation of T cells, and induction of an antigen-specific response (15, 75, 76). Upon maturation, DCs migrate to a lymph node, where they prime and activate antigen/tumor-specific T cells. Subsequently, T helper (Th) or cytotoxic T cells (CTL), migrate into the tumor site where they can perform their effector functions (15, 79).

Additionally, tumor-infiltrating DCs (TIDCs) have been reported to regulate the magnitude and duration of T cell responses within the TME, either through direct antigen presentation or establishment of a favorable cytokine environment, in different tumor models including breast cancer and melanoma (15, 20, 79–84). Antigen presentation within tumors might occur in ectopic tertiary lymphoid structures (TLS), which are hypothesized to play an important role in response to neoantigens that form during later stages of tumor progression (15). It seems that in the cancer setting, tumor-draining lymph node DCs might initially prime naïve T cells, while later intra-tumoral DCs further license and activate T cells in the tumor bed (85). Overall, these studies indicate that TIDCs are required for recruitment, re-priming, and re-stimulation of T cells to acquire full effector function in the TME (20).

DCs have an additional role in innate anti-tumor immunity, through modulation and enhancement of NK cell activity. On the one hand, mature DCs potentiate NK cytotoxicity against tumor cells by secreting pro-inflammatory cytokines (e.g., IL-12, IL15) and cell-to-cell contact. On the other hand, NK cells promote DC infiltration into tumors, as well as their maturation, cytokine-producing ability, migratory potential, and facilitate cross-presentation through the secretion of chemokines and growth factors such as CCL5, XCL1, and FLT3L. This interaction in turn results in enhanced and stronger anti-tumor T cell activation. The dynamic crosstalk between DC and NK takes place in both tumor-draining lymph nodes and in the TME (74, 86, 87). In fact, an optimal anti-tumor immune response appears to rely on effector T cells and NK cells, which are jointly induced and coordinated by DCs (86). DCs are thus essential mediators for the induction of powerful immune responses against cancer cells (88).

Notably, DCs encompass a highly complex and heterogenous population. Regarding their origin and differentiation pathway, four major lineages can be defined: myeloid or conventional DCs (cDC1 and cDC2), plasmacytoid DCs (pDC), inflammatory or monocyte-derived DCs (MoDC), and Langerhans cells (LC). Even though all DCs harbor antigen-presenting and T cell activating abilities, the different subsets present distinct phenotypes and specialized functions. This expands the range and flexibility of immune responses (21, 73, 89). For instance, cDC1 are specialized in cross-presentation and CD8+ T cell responses, cDC2 in CD4+ T cell priming, pDCs in type I interferon-mediated responses, and MoDCs perform different functions in inflammatory settings (15, 21, 89–93).

As such, the immunogenic or tolerogenic functions of DCs, T cell priming or tolerance, depend on their functional subset and their maturation status, which is dictated by environmental cues (75, 77, 79, 88, 94). The phenotypic and functional plasticity of DCs renders them susceptible targets for the evolution of tumor-mediated suppressive mechanisms (95).

TUMOR-INDUCED DC DYSFUNCTION

DCs can play either a regulatory, tolerogenic function or coordinate potent immune responses, depending on the local tumor milieu. Tumors take advantage of this functional plasticity by interfering with DC functions and shifting the balance towards immune evasion (22). As such, tumors employ a variety of mechanisms to disrupt DC functions, mainly mediated through the immunosuppressive TME, that compromise the development of anti-tumor immune responses and facilitate local and metastatic progression (79).

Quantitative and functional impairments of circulating and intra-tumoral DCs have been widely observed in several types of malignancies including melanoma, breast, pancreatic, ovarian, colorectal, prostate, and lung cancer (16). Several studies, have elucidated on escape mechanisms employed by tumors to disrupt DC functions at different levels (**Figure 1**):

1) Impairing proper differentiation of DCs from hematopoietic and myeloid precursors leading to decreased local and circulating DC numbers. Instead, tumors favor differentiation of precursors into immunosuppressive populations, such as MDSCs, TAMs, and BDCA1+CD14+ cells, which further contribute to an immunosuppressive environment (23, 73, 96–104). 2) Inducing apoptosis or favoring exclusion of mature DCs from the TME, for instance, by blocking NK-mediated recruitment of DCs (74, 105). Furthermore, inhibiting proper DC maturation and activation, preventing expression of co-stimulatory molecules, and secretion of pro-inflammatory cytokines, blocking DCs at an immature state. Several tumor-derived factors have been shown to mediate DC defects including TGF-β, TNF-α, IDO-1, PGE2, IL-6, IL-10, VEGF, and GM-CSF (70, 106–116). These factors hinder DC migration, antigen presentation, and effective T cell and NK activation (105–108, 113, 116–128). 3) Directly and indirectly inhibiting anti-tumor T cell functions. The TME skews DCs from immunostimulatory into tolerogenic and immunosuppressive phenotypes and functions, affecting T cell survival and proliferation, or by inducing anergy (an hyporesponsive state) and the expansion of Treg cells. These processes are characterized by the increased expression of immunosuppressive factors such as TGF-β, IL-10, PGE2, IDO-1, and PD-L1 by DCs (104, 114, 115, 129–142).

Overall, the presence of an immunosuppressive TME induces DC-mediated tolerance rather than immunity, contributing to immune escape and dampening of anti-tumor T cell responses. In different studies, these effects on DCs have translated into accelerated tumor progression, increased tumor-draining lymph node metastasis, immunotherapy failure, systemic dysfunctional immune status, and poor prognosis (102, 116, 120, 143–146). In summary, evidence suggests that tumor-induced DC defects are decisive for tumor growth, metastasis initiation, and prognosis. This emphasizes the impact of a phenotype shift in DCs in driving either immunosurveillance or accelerated malignant growth.

NUMERICAL AND FUNCTIONAL DEFECTS OF DCS IN CRC PATIENTS

Given the existence of different DC subsets and functional states, their plasticity in regard to signals from the TME and the

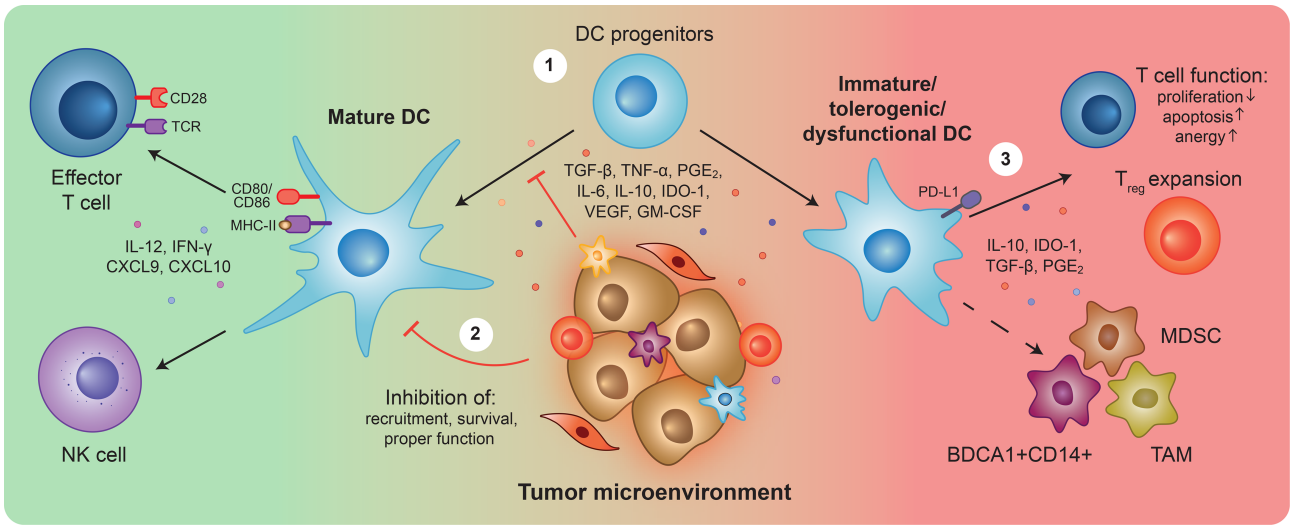

FIGURE 1 | Overview of Dendritic cell (dys)functions in cancer. Upon detection of tumor antigens and danger signals, dendritic cells (DCs) become activated, upregulate co-stimulatory surface molecules and secrete pro-inflammatory cytokines. Mature DCs can (cross)-present antigens, trigger tumor-specific T cell responses, and stimulate natural killer (NK) cell activity to unleash cytotoxic anti-tumor immunity (left). During tumor development and progression, the release of tumor-derived suppressive factors prevents DC progenitors from properly differentiating ①, and differentiated DCs from fulfilling their functions ②. Resulting immature, tolerogenic and/or dysfunctional DCs, characterized by the expression of TGF-β, IL-10, IDO-1, PGE2, and PD-L1, can inhibit T cell anti-tumor responses ③. Furthermore, they can differentiate into and favor the expansion of immunosuppressive populations such as myeloid-derived suppressor cells (MDSCs), BDCA1+CD14+ cells, and tumor-associated macrophages (TAMs). Overall, the impairment of DCs is a crucial step for tumor immune evasion, triggering a cascade of immunosuppression that hampers anti-tumor immunity and creates a propitious environment for tumor growth and metastasis initiation.

consequent impact on anti-tumor immunity, it is not surprising that DC phenotypical changes and defects have shown clinical relevance across different tumor types (22, 147, 148). DCs have been widely investigated in CRC patients with variations in number, phenotype, and function of both circulating and TIDCs reported (**Table 1**).

Several studies have investigated the prognostic value and distribution pattern of TIDCs in CRC patients' tissues. DCs have been linked to both positive and negative effects on CRC prognosis, depending on their maturation status, location, and interaction with other immune tumor-infiltrating cells. Several studies have correlated a higher number of TIDCs with increased patient survival, lymphocyte infiltration, lower metastasis, and overall better prognosis (66, 149–154). In some of these studies, the S100 marker alone was used to identify DCs. These findings may be somewhat limited since S100 expression is restricted to only a few DC subsets, and is not a DC-specific marker being also expressed by other cell types including macrophages (183). Moreover, in these studies the maturation state of DCs was not assessed, which precludes information on DC pro- or anti-tumorigenic polarization and functions.

Further studies have investigated TIDCs distribution in CRC in correlation with their maturation status. Two studies report that the density of tumor-infiltrating mature DCs (mDCs) is lower in metastatic sites than in primary sites, which in turn is lower than in normal mucosa (158, 167). In addition, different studies have shown that mDCs are usually present in the invasive margin and cluster with T cells in lymphoid structures (TLS), whereas immature DCs (iDCs) are often more scattered through the tumor stoma (159, 160). These results suggest differential immune landscapes in primary and metastatic tumor sites and overall mDC exclusion from tumor sites.

Additionally, the maturation status of TIDCs has been correlated with disease progression and patient prognosis. Lower levels of mDC infiltrates have been linked to more advanced disease stage, higher metastatic burden, Treg infiltration, and poor prognosis. Conversely, higher levels of mDCs relative to iDCs, have been associated with stronger Th and CTL responses and better prognosis in general (19, 56, 165, 166, 168–171). These results are in agreement with the anti-tumorigenic potential of mDCs and the tolerogenic role of iDCs. In line with this, MSI tumors with better survival are characterized by an increase in mDCs and lower numbers of Tregs in comparison with MSS (163, 164). Two studies have shown somewhat contradicting results, implying a correlation between increased mDCs infiltration, and shorter survival and increased metastasis (172, 173). More recently, PD-L1+ DCs were clearly associated with CD8+ T cell infiltration and good survival in CRC (174). Interestingly, several studies have linked the observed defects of DCs and poorer survival with increased expression of COX-2, HMGB1, IL-6, and TGF-β by CRC (56, 66, 153, 161, 162, 167). Of note, a limitation of many of these studies is the use of a small set, and often non-DC specific markers to characterize DCs and their maturation status. Notwithstanding, these studies certainly provide valuable insight on TIDCs distribution and prognostic value in CRC patients.

Besides TIDCs, numerical and functional defects of circulating DCs in CRC patients have also been observed. In general and in relation to disease progression, a decreased

TABLE 1 | Overview of studies investigating tumor-infiltrating (TIDCs) and circulating dendritic cells (DCs) in colorectal cancer (CRC) patients.

CRC (n)	Experimental setup	DC characterization	Key conclusions	Reference
TIDCs – interactions with other immune cells and correlations with disease progression and prognosis				
121	Tissue IHC	S100	↑ S100+ DCs ↔ good prognosis, higher survival, often without metastasis and ↑ lymphocyte infiltration	(Ambe, Mori, & Enjoji, 1989) (149)
30	Tissue IHC	S100	↑ S100+ DCs ↔ good prognosis ↓ S100 +DCs ↔ lymph node and hepatic metastasis, >stage III	(Nakayama et al., 2003) (150)
104	Tissue IHC	S100 and HLA-II	↑ S100+ DCs ↔ ↑ T cell infiltration and disease-free survival	(Dadabayev et al., 2004) (151)
40	Tissue IHC	S100, CD11c, CD208, CD209, CD123, and CD1a	S100+ DCs ↔ Tregs ↑ S100+ DCs ↔ prolonged survival ↓ S100+ DCs ↔ worse prognosis	(Nagorsen et al., 2007) (152)
16	Tissue IHC	CD205	↓ CD205+ DCs and high HMGB1 expression by CRC ↔ lymph node metastasis	(Kusume et al., 2009) (153)
52	Tissue IHC	CD11c+	↓ CD11c+ myeloid DCs ↑ Tregs ↔ tumor invasion, advanced stage, lymph node metastasis and poor prognosis	(Gai, Li, Song, Lei, & Yang, 2013) (154)
63	Tissue IHC	CD123 (pDCs)	↑ pDC/myeloid DC ratio and ↑ Tregs ↔ lymph node metastasis	(Gai, Song, Li, Lei, & Yang, 2013) (155)
149	Tissue IHC	BDCA-2+ (pDCs)	↑ pDC ↔ TLS and prolonged survival	(Kießler et al., 2021) (156)
58	Flow cytometry and RNA sequencing	BDCA-2+ (pDCs)	↑ pDC ↓innate lymphoid cells ↔ advanced disease stage	(Wu et al., 2021) (157)
TIDCs – maturation status and distribution				
57	Tissue IHC	CD83, HLA-DR, CD40, and CD86	Density of mDCs: Normal mucosa > primary CRC > metastatic CRC No association with TGF-β or IL-10	(Schwaab, Weiss, Schned, & Barth, 2001) (158)
17	Tissue IHC	CD83 and CD1a	CD83+ mDCs: present in the invasive margin and cluster with T cells CD1a+ iDCs: scattered in the tumor stroma	(Suzuki et al., 2002) (159)
60	Tissue IHC	CD1a, S100, CD83, and HLA-DR	CD83+ mDCs: present around metastases and in the sinusoidal lumen CD1a+ iDCs: scattered in the tumor stroma	(M. Gulubova, Manolova, Cirovski, & Sivrev, 2008) (160)
26	Tissue IHC and gene expression	CD83	Primary site and lymph nodes: ↓ CD83+ mDCs ↔ high COX2 and IL-6	(Cui et al., 2007) (161)
23	Tissue IHC and gene expression	CD1a, CD83, and CD208	↓ CD83+ CD208+ mDCs ↑ CD1a+ iDCs ↔ increasing COX2 expression	(Yuan et al., 2008) (162)
69	Tissue IHC	S100, CD208	In MSI tumors in comparison with MSS: ↑ CD208+ mDCs and ↓ Tregs	(Bauer et al., 2011) (163)
133	Tissue Gene expression	Genes implicated in immune response	In MSI tumors in comparison with MSS: ↑ co-stimulatory molecules in DCs	(Banerjea et al., 2004) (164)
TIDCs – maturation status and correlations with disease progression and prognosis				
70	Tissue IHC	CD83	↓ CD83+ mDCs ↔ poor prognosis	(Miyagawa et al., 2004) (165)
22	Tissue IHC	CD83	↓ CD83+ mDCs ↔ advanced disease and lymph node metastasis ↑ CD83+ mDCs and IL-12 expression ↔ better prognosis	(Inoeu et al., 2005) (166)
142	Tissue IHC	HLA-DR, CD1a, and CD83	↓ CD83+ mDCs ↔ shorter survival ↔ TGF-β expression by CRC	(Maya Gulubova et al., 2010) (56)
86	Tissue IHC	HLA-DR, CD1a, and CD83	Metastasis in comparison to metastasis-free samples: ↓ CD83 + mDCs and ↑TGF-β	(Maya Gulubova et al., 2013) (167)
44	Tissue IHC	CD1a and DC-LAMP	↓TILs ↑ CD1a+ iDCs/DC-LAMP+ mDCs ratio and KRAS mutation ↔ higher risk of disease recurrence	(Kocián et al., 2011) (168)
145	Tissue IHC	CD1a, S100, CD83, and HLA-DR	↓ CD83+ HLA-DR+ mDCs in invasive margin ↔ advanced stage (metastasis) and worse prognosis	(Maya V. Gulubova et al., 2012) (169)

(Continued)

TABLE 1 | Continued

CRC (n)	Experimental setup	DC characterization	Key conclusions	Reference
556	Gene expression	Several DC-related genes	↑ mDCs ↑ T cells ↔ low risk group	(M. Li et al., 2020) (170)
473	Gene expression	CD80, CD83, and CD86	↑ CD80+, CD83+, CD86+ mDCs ↔ CXCL8 expression by CRC	(E. Li et al., 2021) (171)
326	Gene expression	Several DC-related genes	↑ DCs, IL-12 and in TLS ↔ strong Th1 and CTL response and more favorable prognostic	(Coppola et al., 2011) (19)
104	Tissue IHC	S100, CD1a, CD208, and HLA- II	↑CD208+ mDCs in the stroma ↔ shorter overall survival ↑CD1a+ iDCs in the advancing margin ↔ shorter disease-free survival	(Sandel et al., 2005) (172)
71	Tissue IHC	CD83	↑ mDCs ↔ tumor invasion and lymph node metastasis	(Pryczynicz et al., 2016) (173)
221	Tissue IHC	CD11c and PD-L1	↑ CD11c+ PD-L1+ DCs ↔ good survival and ↑ CD8+ T cell density	(Miller et al., 2021) (174)
Blood circulating DCs - Numerical defects				
106	Flow cytometry	HLA-DR and CD86	↓ Circulating DC ↔ ↑ TGF-β levels	(Huang et al., 2003) (175)
54	Flow cytometry	HLA-DR, CD11c, CD83, and CD86	Numerical and functional impairment of DC progenitors ↔ stage of the disease and ↑ VEGF levels	(Della Porta et al., 2005) (176)
27	Flow cytometry	BDCA-1, BDCA-2, BDCA-3, CD80, CD86, and HLA-DR	DCs number: healthy > metastatic > non-metastatic > chemotherapy treated subjects	(Bellik et al., 2006) (177)
26	Flow cytometry	CD33 and CD123	↓ CD123+ pDCs ↔ advanced stage	(Orsini et al., 2014) (178)
Blood circulating DCs - Functional defects				
31	Flow cytometry, functional assays	CD11c, CD123 HLA-DR, CD80, CD86, and CD83	↑ immature myeloid cell progenitors Defective DC maturation *ex vivo* ↔ ↑ VEGF Anti-VEGF antibody treatment: ↑ *ex vivo* stimulatory capacity of DC ↔ ↑ antigen-specific allogenic T cell proliferation	(Osada et al., 2008) (179)
23	Flow cytometry, functional assays	CD40, CD80, and CD83	Defective generation of mature and functional DC *ex vivo* ↔ advanced disease stage ↓ Ability to present antigens to allogeneic T cells ↑IL-10 ↓IL-12 and TNF-α	(Orsini et al., 2013) (180)
16	Flow cytometry, functional assays	CD83 CD1a HLA-DR CD86 FITC, CD80, CD209, and CD206	Defective DC maturation *ex vivo*	(Maciejewski et al., 2013) (181)
30	Flow cytometry, functional assays	CD80, CD11c, HLA-ABC, HLA-DR, CD14, CD133, CD11b, CD209, and CD86	Defective DC maturation *ex vivo* ↓IL-12	(Hsu et al., 2018) (182)

↔: correlation/association, ↑ higher infiltration/higher density/increase, ↓ lower infiltration/lower density/decrease. IHC, immunohistochemistry; mDCs, mature DCs; iDCs, immature DCs.

number of circulating DCs, increased number of progenitors, and a higher iDC/mDC ratio in CRC patients have been reported (175–179). These defects have been associated with increased serum levels of TGF-β and VEGF (175, 176, 179). Furthermore, functional defects have been noted, including defective *ex vivo* differentiation and maturation of DCs from monocytes, tolerogenic phenotypes with decreased IL-12 and TNF-α release, increased release of IL-10 and TGF-β, and a compromised ability to induce allogenic T cell proliferation (179–182). These findings highlight the importance of systemic immunosuppression exerted by the CRC.

In addition, several studies have concluded that CRC explant tissue-conditioned medium inhibits LPS-induced *in vitro* DC maturation and function. In these assays, upregulation of co-stimulatory markers (CD80 and CD86) and PD-L1, and secretion of IL-12 and TNF-α was inhibited, while secretion of IL-10 was potentiated suggesting DCs acquire a tolerogenic phenotype (184–187). One study even correlated stronger inhibition of DC maturation by CRC-conditioned medium with poorer survival in patients (186). A variety of tumor-derived factors secreted by CRC including VEGF, CCL2, CXCL1, and CXCL5, were shown to mediate these effects synergistically (184, 185). Collectively, these studies demonstrate that CRC, mainly through soluble mediators, evades anti-tumor responses by exerting local and systemic immunosuppression and disabling both infiltrating and circulating DCs.

Strikingly, very few studies have focused on the different DC subsets, which present different functional specializations, in relation to CRC and to T cell function. For instance, infiltration by pDCs has yielded controversial results, with some studies associating pDCs infiltration with Treg development and poorer prognosis and others with increased survival, but without taking maturation status into account (155–157).

All in all, the reported findings illustrate the importance of local and systemic modulation of DCs in CRC. Currently, there are insufficient available data to elucidate on the complex mechanisms underlying DC dysfunction in CRC patients. To develop a more comprehensive picture of the implications of DC dysfunction in CRC, additional studies will be needed to determine the differential roles of the DC subsets in CRC, taking into account their functional specialization, maturation status and plasticity, which can have contrary impacts on tumor progression and prognosis (73). Moreover, further insight on the

differences between metastatic and primary tumor sites will be of value.

TUMOR-INDUCED DC DYSFUNCTION AND IMMUNOTHERAPY EFFICACY

As illustrated in the previous section, the CRC immunosuppressive TME shifts the delicate balance of DCs from inflammation to tolerance, fueling disease progression and spread. In addition, DC dysregulation has also been implicated in patients' unresponsiveness to immunotherapies, further contributing to a poor prognosis. Current immunotherapeutic approaches for CRC have been mainly focused on targeting T cells, either by immune checkpoint inhibitors or by stimulating T cell activating receptors (188). However, only CRC patients harboring tumors with high mutational burden - MSI, accounting for less than 5% of the patients with mCRC - benefit from these treatments (35, 36).

Interestingly, in MSS CRC patients tumor-infiltrating neoantigen-specific T cells have been detected despite their low mutational burden and low responsiveness to immune checkpoint inhibitors (189). This and other data suggest that, upon treatment, tumor-specific T cells can be generated but are not functional (47, 189, 190). This low T cell reactivity has been linked to a TGF-β-rich TME (189). Importantly, this T cell-centric approach of immunotherapy does not account for DC impairments, despite their crucial role in T cell priming, activation, and recruitment in the tumor bed.

Indeed, several studies have emphasized a strong dependency of effective immune checkpoint inhibition on correctly functioning and activated TIDCs. These studies confirm that cross-priming, licensing, and recruitment of T cells by functional intratumoral DCs is required for successful responses to anti-PD-1 therapy and T cell adoptive therapy. And, that this is mainly mediated by CXCL9/10, IL-12, and IFN-γ secretion by DCs (85, 123, 191–195).

In line with this, one study in a melanoma model has shown that expansion and activation of TIDCs at the tumor site by recruiting and activating agents such as FLT3L and poly I:C, enhanced therapeutic response to immune checkpoint inhibitors (196). In addition, a recent study indicates that blocking CRC-induced WNT2 secretion by CAFs restores DC functions enhancing anti-PD-1 efficacy (197). These studies emphasize the importance of functional DCs in effective intra-tumoral DC-T cell crosstalk for immunotherapy response. Therefore, targeting T cells without taking into account and resolving DC dysfunction might hamper the success of T cell-centered immunotherapies in CRC (198, 199).

Additionally, other studies show that NK cell and DC reciprocal interactions are required for enhanced T cell responses and determine responsiveness to immune checkpoint inhibition treatment (200, 201). NK cell frequency correlates with enhanced DC infiltration in the tumor, which in turn correlates with patient responsiveness to immune checkpoint inhibitors and increased survival. Moreover, studies have shown that DC vaccine efficacy is strongly dependent on NK cell activity and DC-NK cell crosstalk (202–204). This highlights the importance of NK cells in tumor immunity not only by their ability to kill cancer cells directly, but also as promoters of DC activity.

In the clinic, studies with DC vaccines and other DC-targeting therapies in CRC have yielded modest results. DC vaccines consisting of *ex vivo* activated patient DCs, tailored against tumor-associated antigens, have the potential to trigger and boost T cell anti-tumor responses. This highly specific approach, combined with a relatively low risk of toxicity, makes DC vaccines particularly promising (10, 205). However, despite initial encouraging results including increased T cell responses and a good safety profile, DC vaccines have not shown strong therapeutic benefit in CRC patients (206–216). This is hypothesized to be linked to the strongly suppressive TME, particularly a TGF-β rich TME (10, 217, 218) and, consequently, defective and immunosuppressive DC populations. Besides suppressing administered DCs, these dysfunctional DC subsets can also limit T cell infiltration and effector function (96, 219). Current studies focus on improving vaccine platforms, increasing and expanding tumor specificity of vaccines, counteracting the host immunosuppressive mechanisms of resistance, and testing combinatorial therapies (220). Indeed, three ongoing or recently completed trials for mCRC aim to synergize DC vaccines with IL-2 (NCT02919644) or with immunological checkpoint inhibitors (NCT04912765 and NCT03152565). In conclusion, it seems that efficacy of immunotherapies is reliant on functional DCs for proper T cell-DC-NK cell crosstalk, which is disrupted by the strongly immunosuppressive TME.

REVERTING DC DYSFUNCTION TO UNLEASH ANTI-TUMOR IMMUNITY AND RESPONSE TO IMMUNOTHERAPY

Mounting evidence highlights the central role of DCs in anti-tumor immunity and consequently on immunotherapeutic responsiveness and urges the development of DC- and TME-targeted therapies to tackle DC dysfunction in treatment-resistant CRC patients. Unleashing DCs emerges as a crucial step to make immune checkpoint inhibition and other immunotherapies available to all CRC patients. The development of combinatorial therapies for mCRC is being actively sought since monotherapies have not demonstrated effectiveness in improving patient's outcome (10). Since tumors evolved multiple mechanisms to avoid immune evasion, a multi-faceted approach focusing on different mechanisms will most likely be needed to address current issues in CRC treatment (10, 199, 221–223).

To breach the strong immunosuppressive CRC microenvironment, a promising therapeutic venue seems to include combinatorial strategies to in concert: 1) foster DC activation and function, boosting antigen presentation or TIDC abundance, 2) stimulate and unleash NK or T cells by immune checkpoint inhibition, and 3) target tumor-mediated mechanisms and tumor-released immunosuppressive factors. This should in principle allow overcoming of the strong suppressive TME, trigger more efficient NK-DC-T cell crosstalk and lead to full unleashing of local and systemic anti-tumor immune responses. As a metaphor this

approach can be described as not only releasing the brakes of the immune system by lifting the vail of immunosuppression but also pressing the gas pedal by stimulating the key coordinators and effectors of immune responses.

The main aim of DC-targeting strategies is to skew the TIDC phenotype from tolerogenic to inflammatory, and enhance DC-intrinsic abilities. Strategies that aim to restore and stimulate DC functions, although not powerful in the clinic alone, might have a key role in combinatorial treatments (10, 224–228). Approaches to circumvent tumor-mediated DC dysfunction can consist of DC vaccines or directly targeting and stimulating DCs *in situ*, by delivering DC-recruiting or promoting agents such as FLT3L, CpG, TLR and STING agonists (199). In addition, reverting DC dysfunction would break the positive feedback loop of immunosuppression, allowing wider reprogramming of the TME.

Thus far, overcoming the TME remains the most important and daunting challenge for CRC. Despite promising leads on the apparent key role of tumor-released suppressive factors such as TGF-β, VEGF, and PGE2, it is still largely unclear how CRC shapes DC fate. A more complete understanding of the complex web of interactions and elucidation on key mechanisms in play between CRC and DCs is required. Future studies will certainly provide new rationales and open doors for the design of novel therapies to unlock the full anti-tumor potential of DCs, while sensitizing previously unresponsive patients to immunotherapy.

FUTURE PERSPECTIVES

There is abundant room for further progress in understanding CRC-DC interactions. In future studies, it is important to consider both the functional and phenotypical plasticity of DCs subsets, and the heterogeneity and complexity of CRC. It is well-established that different DC subsets can have distinct, either complementary or opposing, functions in anti-tumor immunity and hence affect tumor progression differently (73). To better dissect this heterogeneity, further research is required to characterize the functional status, quantify, and assess the distribution of the different DC populations present in the tumor sites and in circulation in CRC patients. Whether these are correlated to CRC molecular subtype, disease progression, prognosis, treatment response or immunosuppressive systemic factors needs to be determined. Possibly, different subsets have different predictive potential and might arise as novel biomarkers for disease progression and treatment response. To get a comprehensive overview, patient material from different disease stages and molecular subtypes, including tissue sections, fresh biopsies and blood samples, will be valuable.

Furthermore, future research should aim to explore and study both metastatic and primary sites. There are few studies with a comparative perspective lens between primary and metastatic CRC. It is important to address differences in DCs infiltration, phenotype and functionality, and tumor-mediated evasion mechanisms. Metastatic sites are often different not only at a molecular level but also in the immune landscape and the TME. Moreover, it is important to research metastases since these are often more resistant to therapy than their primary counterparts. As such, investigating and tackling the immunosuppressive environment of not only the primary tumor, but also the metastatic sites is of outermost importance (229).

At the molecular level, it is also important to gain insight on how CRC shapes the different DC subsets, what are the underlying mechanisms, and what are the local mediators of DC dysfunction. Also, many questions remain unanswered regarding how the different subsets correlate with each other and with T and NK cell effector function in CRC. To study in more detail these CRC-specific underlying molecular mechanisms, *in vitro* models are most suitable. Currently, there is a shortage of relevant and representative *in vitro* models to study the CRC TME.

Recently, patient-derived organoids (PDOs) are emerging as a powerful tool to study CRC heterogeneity and therapy responses by faithfully recapitulating many of the traits of patients' disease. Moreover, studying DCs in a tumor organoid context is still a largely unexplored field with a lot of opportunities. As such, 3D co-culture systems of DCs and CRC PDOs seem a promising approach to more closely study their interactions. In order to more representatively model the biological context and the *in vivo* interactions, higher complexity 3D co-culture models including stroma cells and different immune infiltrates that mimic the complex structure and composition of a tumor and its microenvironment are sought after (104, 230–232). In this line of research, recently a complex organotypic skin model was successfully developed to study DCs in melanoma (104). Hopefully in the near future, similar organotypic or complex 3D organoid based co-culture systems can be developed to study the CRC TME, both primary and metastatic, and follow the behavior of different subsets of DCs.

In addition, these models can possibly achieve sufficient physiological relevance to serve as testing platforms for novel therapies. This would be valuable since there are many unexplored combinatorial opportunities including TME- and DC-targeting therapies for CRC. It also remains to be determined which DC targeting strategies are effective and synergize with other immunotherapies in CRC patients. Furthermore, co-culture systems with PDOs offer the opportunity to test and tailor combinatorial strategies in a patient-specific manner.

CONCLUSIONS

Metastatic CRC remains one of the most aggressive and lethal cancers, with the large majority of patients being refractory to therapy. Disease aggressiveness and resistance to therapy has been linked to the tumor genetic makeup and a highly immunosuppressive TME. DCs have key roles in anti-tumor immunity, making them crucial targets for tumor evasion mechanisms. Overall, literature suggests that CRC-induced DC dysfunction is decisive for: impairing anti-tumor immune responses, tumor progression, metastatic colonization and

initiation, and unresponsiveness to immunotherapies such as immune checkpoint blockage.

Unravelling the complex crosstalk between CRC and DCs and determining its significance for patients holds promise for identifying and modulating key mechanisms involved in disease progression. This opens doors for the design of novel strategies to reverse DC dysfunction. In principle, restoring DC functions can unlock the full anti-tumor potential of DCs and hence, unleash systemic anti-tumor immunity mediated by T and NK cells against primary and metastatic CRC. This approach should make immunotherapies available for more patients. Therefore, reverting DC dysfunction emerges as a promising path for CRC treatment and a critical pillar for combinatorial strategies. In order to design novel therapies, a completer and more comprehensive overview of the CRC TME and the mechanisms driving tumor progression and induction of DC tolerizing properties is necessary. For futures studies, examining patients' tissues and blood and development of *in vitro* TME co-culture models based on PDOs appear as promising tools to obtain the missing knowledge.

AUTHOR CONTRIBUTIONS

BS performed literature searches and wrote the first version. All authors contributed to the article and approved the submitted version.

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Transcriptomic Analysis Identifies A Tolerogenic Dendritic Cell Signature

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Dendritic cells (DC) are central to regulating innate and adaptive immune responses. Strategies that modify DC function provide new therapeutic opportunities in autoimmune diseases and transplantation. Current pharmacological approaches can alter DC phenotype to induce tolerogenic DC (tolDC), a maturation-resistant DC subset capable of directing a regulatory immune response that are being explored in current clinical trials. The classical phenotypic characterization of tolDC is limited to cell-surface marker expression and anti-inflammatory cytokine production, although these are not specific. TolDC may be better defined using gene signatures, but there is no consensus definition regarding genotypic markers. We address this shortcoming by analyzing available transcriptomic data to yield an independent set of differentially expressed genes that characterize human tolDC. We validate this transcriptomic signature and also explore gene differences according to the method of tolDC generation. As well as establishing a novel characterization of tolDC, we interrogated its translational utility *in vivo*, demonstrating this geneset was enriched in the liver, a known tolerogenic organ. Our gene signature will potentially provide greater understanding regarding transcriptional regulators of tolerance and allow researchers to standardize identification of tolDC used for cellular therapy in clinical trials.

Keywords: dendritic cell, tolerogenic dendritic cell (tolDC), gene expression profile analysis, mature dendritic cells, mononuclear phagocyte cells, transcriptomic, liver, human dendritic cell

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INTRODUCTION

Dendritic cells (DC) represent a population of bone marrow (BM)-derived cells responsible for the collection and presentation of captured antigen (Ag) (1). DC are found throughout the body, and their capacity for Ag presentation provides a crucial link between innate and adaptive immune responses. Multiple DC subsets have been described, broadly divided into myeloid and plasmacytoid groups (2). Similar to other immune cells, DC are also able to alter their phenotype and function based on environmental cues (3), contextual inflammatory signaling, and the presence

of self/non-self Ag. Classically, mature DC drive effector T cell responses, and immature DC mediate central or peripheral tolerance primarily through immunoregulatory factors that induce regulatory or anergic T cells (4). An additional subset that are maturation-resistant – so-called tolerogenic DC (tolDC) – can be manufactured *ex vivo* but have not yet been found physiologically. TolDC have been extensively interrogated in pre-clinical models, and are exceedingly effective at limiting host immune responses that drive autoimmune disease [summarized in (5)] or allograft rejection in transplantation [summarized in (6)]. Capitalizing on their ability to modulate T and/or B cell behavior and release immunomodulatory molecules, tolDC have been used in recent phase I/II clinical trials for type 1 diabetes (7), rheumatoid arthritis (8), multiple sclerosis (9), and liver and kidney transplantation (10) as therapeutic agents that reduce exposure to non-specific immunosuppressive drugs.

Multiple protocols for the generation of tolDC exist (11). BM-derived progenitors (animals) and CD14+ peripheral blood mononuclear cells (PBMC, humans) are driven towards prototypic DC using growth factor/cytokine cocktails, and then "tolerized" pharmacologically. Interleukin-10 (IL-10) and vitamin D-based regimens are most frequently used, a substantial list of pharmacological modifiers of DC function exists (12) which continues to expand (6). Avoiding *ex vivo* isolation and manipulation, *in vivo* modulation using DC-specific targeting techniques, such as nanoparticles (13, 14) or antibodies (15), can directly deliver a pharmacological payload. Despite treatment heterogeneity, the DC phenotype is characterized by immunoregulatory properties (16) which then assumes generation of stable tolDC.

Identification of DC subsets is typically based on cell-surface markers. Although expression appears relatively conserved between species, tissues and disease models (2), the same standardized characteristics are not yet available for tolDC. Indeed, tolDC used in recent clinical studies did not have uniform methods for generation, phenotype or functional measurements (17). To date, there is no consensus for "gold-standard" validation of tolerogenic properties, and current methods range from analysis of cell-surface markers to allogeneic T cell stimulation (10). This has significant implications for clinical trials where differences in tolDC generation may impact clinical outcomes. There is also ongoing concern that tolDC are not stably manipulated and, like regulatory T cells, can be subverted to activated or inflammatory forms by a permissive microenvironment. Understanding gene changes that robustly reflect tolDC would be a useful tool in standardizing their generation, which may ultimately impact patient outcome.

Transcriptomic analysis allows for the identification of conserved and differentially expressed genes in tolDC regardless of the method of generation. A specific transcriptomic signature may also assist with discovery of surrogate markers that may be used clinically. The adaptation of differentially expressed genes to enrichment pathways also provides insight into the biological interpretability of gene(s) of interest. Recent literature (18) seeking to bridge this gap in the literature are limited to consolidating already reported signatures of previous studies and drawing on published conclusions to extract a transcriptome unique to the tolDC phenotype. We have addressed this shortcoming by analyzing available datasets to yield an independent set of differentially expressed genes within each study. Comparing these results across datasets yielded a common tolDC transcriptome which we then validated. We used the same pipeline to generate a mature DC transcriptome, and both novel gene signatures were applied to immune cell populations *in vivo*.

METHODS

TolDC Data Acquisition

A search to identify publicly available gene expression data in the Gene Expression Omnibus (GEO) https://www.ncbi.nlm.nih.gov/geo/ was performed using the terms: "tolerogenic dendritic cell", "regulatory dendritic cell" and "tolDC". The search for publications up to December 2020 revealed 136 Datasets, of which 98 were human. Datasets were initially excluded from downstream analysis if they did not have an immature DC phenotype (control) within the dataset. Only 24 were whole datasets, and 8 contained cell samples that included adequately phenotyped tolDC (**Figure 1A**). These datasets were arbitrarily divided into two groups: 5 datasets were used for initial tolDC gene set discovery, and the 3 remaining were used for validation. One further validation dataset was obtained from ArrayExpress (19).

Data Analysis

The raw data of each of the five datasets precured [GSE13762 (20), GSE23371 (21), GSE56017 (22), GSE117946 (23), GSE52894 (24)] were obtained from the gene expression omnibus (https://www.ncbi.nlm.nih.gov/geo/). All five datasets were normalized using the quantile normalization method, with each dataset filtered to exclude genes with nil expression. Within each dataset, differential gene expression analysis was performed using limma (Smyth G. K. 2004) with Benjamini–Hochberg multiple testing correction ($p < 0.05$). In this way, a moderated test statistic was calculated for each gene within each dataset. Moderated test statistics were converted to z-scores, and subsequently p-values, as described in the directPA vignette (25). Pearson's method of combining p-values was used to derive an overall significance score for each gene across all datasets (**Figure 1B**). An overall significance score of $p < 0.00001$ was used as the threshold to establish genes in the tolDC transcriptome.

TolDC Gene Signature Validation

Three (3) datasets acquired from GEO (GSE104438 (26), GSE98480 (27), GSE92852 (28) containing tolDC and immature DC gene expression data were used for validation. A final validation was also performed using data from ArrayExpress database (E-MTAB-6937 (19). As with our discovery and initial validation set, we analysed each dataset

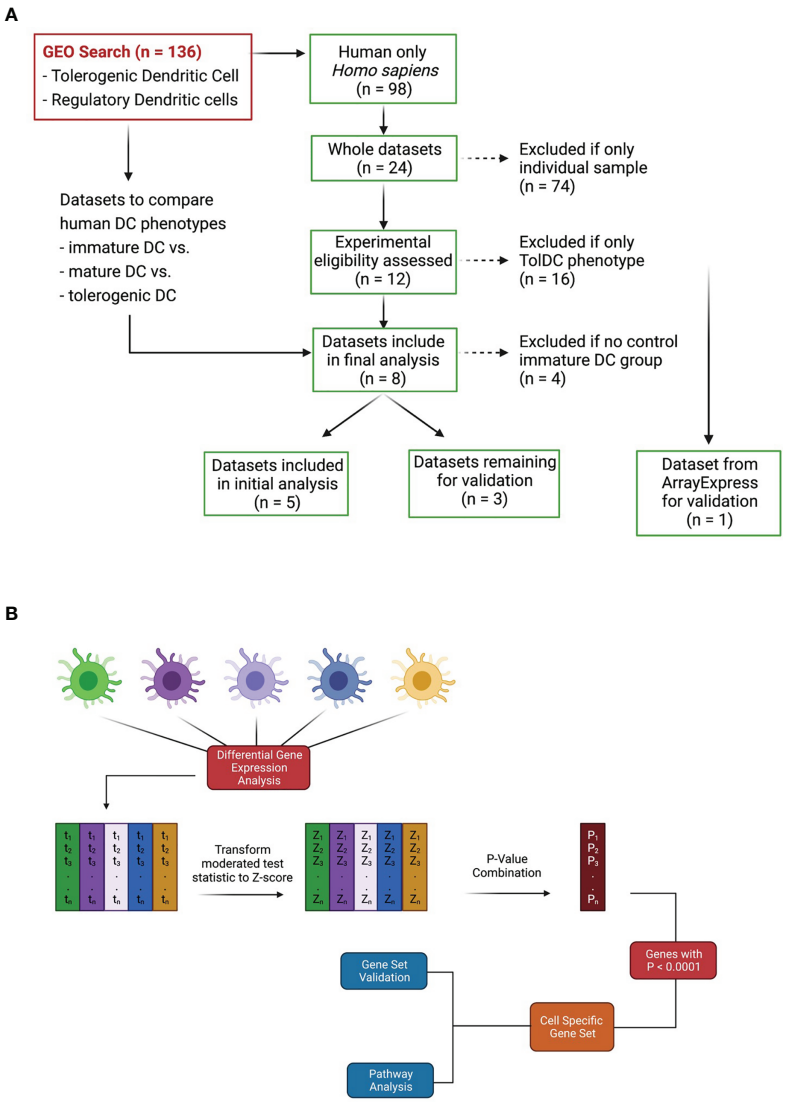

FIGURE 1 | Dataset identification and workflow for tolDC gene analysis. **(A)** Flowchart demonstrating relevant GEO search with inclusion and exclusion criteria. **(B)** Pipeline for generating tolDC, AADC and mature DC gene signatures.

individually to diminish potential batch effects that would arise from merging datasets. In all datasets, the moderated test statistics for each gene were converted into z-scores (as outlined in **Figure 1B**) and the pattern of gene expression compared with our tolDC gene set.

Alternatively Activated Dendritic Cell Gene Signature

In a similar manner to the identification of genes critical to tolDC, we determined genes differentially expressed between the tolDC stimulated with and without lipopolysaccharide (LPS). Three datasets were used in the analysis: GSE23371 (21), GSE117946 (23), GSE52894 (24). Differential gene expression was performed using the limma pipeline optimized as above, combining the results of our analyses using Pearson's Method, and yielding a set of genes critical to defining AADC.

Mature DC Gene Signature

Differentially expressed genes between immature DC stimulated with and without lipopolysaccharide (LPS) were also explored. Four datasets were used in the analysis: GSE23371 (21), GSE56017 (22), GSE117946 (23), GSE52894 (24).

Analysis of Enriched Pathways

A Wilcoxon rank sum test was performed on the combined p-value that was determined for each gene within our gene set analysis, returning a significance value for KEGG pathways that were enriched in the DC of interest. A subsequent Gene Set

Enrichment Analysis (GSEA) was performed on the ranked list of genes, executed using the clusterProfiler (29) package in R.

Signature Validation

We sought to validate the specificity of our mature and tolDC signature using *in vivo* datasets that contained mononuclear phagocytes (MNP), including recognized DC subsets (30, 31) or peripheral blood immune cell subsets [GSE28492 (32)]. RNAseq data was normalized using the TMM method without filtering, microarray data was normalized using quantile normalization, and gene expression was compared between each cell phenotype.

Single Cell RNAseq of Kidney, Liver, and PBMC Datasets

Five individual single cell RNAseq (scRNA-seq) samples were obtained from the Panglao database (https://panglaodb.se/). The search criteria were initially limited to liver tissue only from human donors. The accession code SRA716608 was used to extract scRNAseq into R for analysis. The five samples were normalized and integrated using the harmony algorithm. The combined dataset was then analysed using the Uniform Manifold Approximation and Projection (UMAP) dimensional reduction technique. The tolDC phenotype was then plotted on the UMAP projection. To compare tolDC and mature DC gene signatures in different tissue compartments, liver (SRA716608, n = 22154 cells), peripheral blood mononuclear cells (PBMC, SRA749327, n = 15881 cells) and kidney cortex (SRA598936, n = 3573 cells) scRNA-seq samples were also acquired. Datasets belonging to individual tissue types were integrated using the harmony method, normalized and scaled. The expression of genesets was measured between DC in each tissue type.

Data Availability and Code Statement

Data utilized for this study is publicly available using the GEO accession codes listed. The code utilized to generate analysis and figures is available at: https://github.com/Harry25R/Transcriptomic-analysis-identifies-a-tolerogenic-dendritic-cell-signature.git.

RESULTS

Dataset Quality Control

Five complete datasets with tolDC gene sequencing were retrieved. Each dataset had a different method of tolDC generation and 3 studies shared the same sequencing platform (**Table 1**). A principal component analysis (PCA) identified phenotypic specific differences between samples in the GSE52894 dataset (24) (**Figure 2A**). This was consistent across all included datasets (**Supplementary Figure 1A**). Across the first principal component we observed large differences when DC were matured with LPS. The largest source of variation was between tolerogenic and mature DC, an expected result given the regulatory nature of tolDC compared to mature (immunogenic) DC. Confirming these results, unsupervised hierarchical clustering between samples exhibited strong correlation between samples of the same phenotype (**Supplementary Figure 1B**).

Establishing a tolDC Gene Signature

The results of individual differential gene expression analysis were ranked by p-value. The top 10 up-regulated and downregulated genes are listed in **Tables 2A**, **2B**, respectively. Our results were consistent with previous reports, suggesting no homogeneity in differentially expressed genes DEG between different methods generating tolDC if only looking at the strongest changes (18). By considering more than just the top genes, we then assessed homogeneous differential gene expression across the datasets, identifying 53 genes with a combined p-value$<10^{-5}$ which we deemed to be characteristic of tolDC (**Table 3**). The top 20 DEG are displayed in heatmap form (**Figure 2B**).

TolDC Pathway Enrichment Analysis

Mapping DEG within the tolDC gene set to the KEGG database returned several enriched pathways (**Figure 2C**). The mitogen-activated protein kinase (MAPK) pathway was significantly enriched, as were cyclic AMP, Ras-related Protein 1, Forkhead

TABLE 1 | Identified publicly available gene datasets including immature, tolerogenic and mature DC for initial tolDC gene set discovery.

Dataset ID	Platform ID	References	Sample Proportions	Agent Used to Induce the tolDC Phenotype
GSE13762	GPL570	(20)	4 x imDC, 8 x tolDC	Vitamin D
GSE23371	GPL570	(21)	3 x imDC 3 x imDC + LPS 3 x tolDC 3 x tolDC + LPS	Interleukin 10 & Dexamethasone
GSE56017	GPL570	(22)	6 x imDC 6 x imDC + LPS 6 x imDC + Dexamethasone 6 x tolDC	Dexamethasone
GSE117946	GPL6244	(23)	4 x imDC 4 x imDC + LPS 4 x tolDC 4 x tolDC + LPS	Interleukin 10
GSE52894	GPL10558	(24)	4 x imDC 4 x imDC + LPS 4 x tolDC 4 x tolDC + LPS	Dexamethasone & Vitamin D

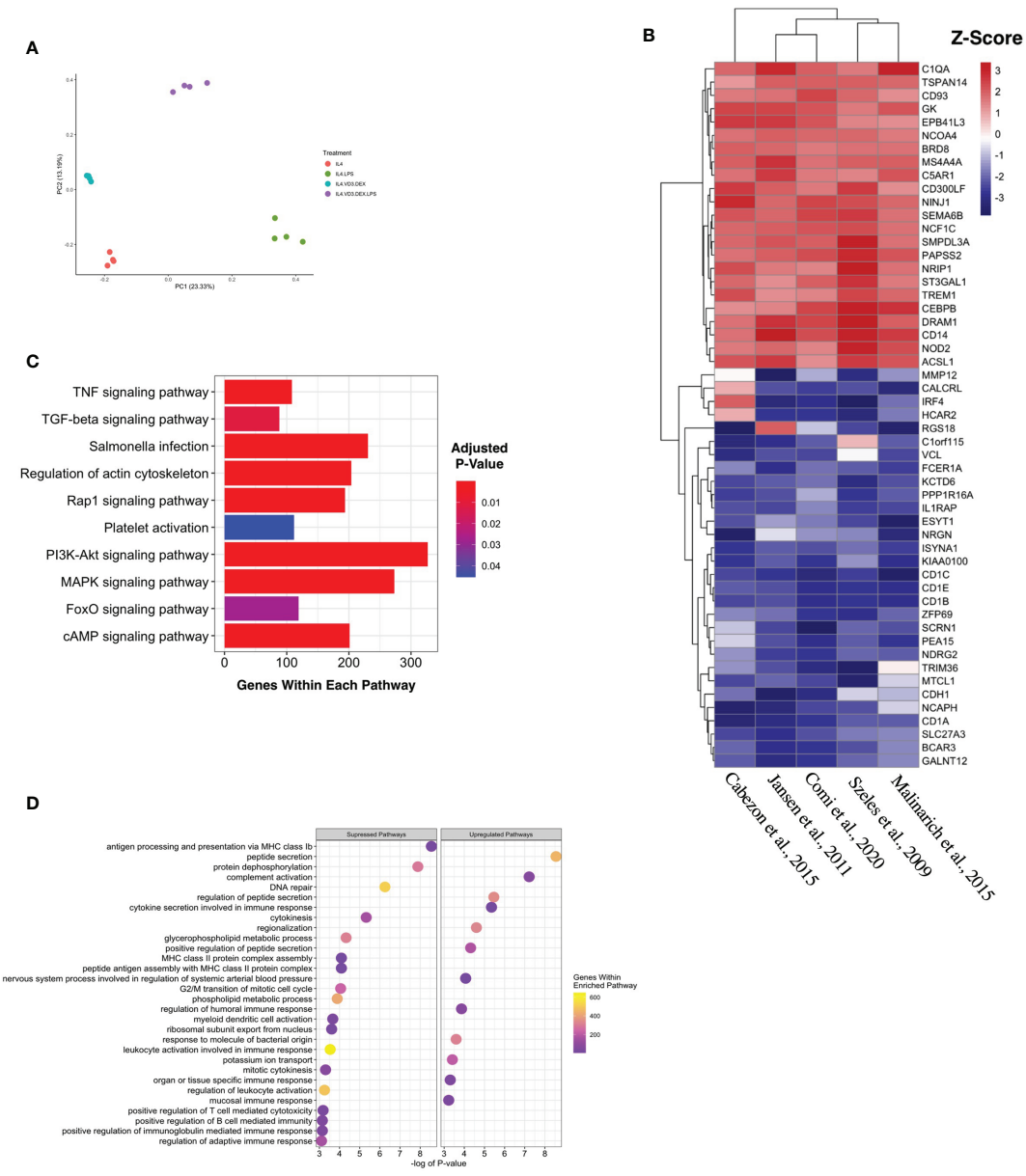

FIGURE 2 | Generating a unique tolDC transcriptome. **(A)** Principal component analysis (PCA) plot characterizing change in gene expression profiles between immature DC (red), mature DC (green), tolDC (blue), or alternatively-activated tolerogenic DC (AADC, purple) in GSE52894. Each dot presents a sample, and each color represents a DC phenotype. **(B)** Heatmap representation of the top 20 differentially expressed genes (DEG) by tolDC. DEG were arranged by hierarchical clustering on the vertical axis. Datasets, also clustered by hierarchical clustering, are displayed on the horizontal axis. The p-value yielded from each study were converted to z-scores and plotted. **(C)** KEGG and **(D)** Gene Set Enrichment analyses. Each point on the dot plot represents the number of genes involved in the relevant pathway. The gene ratio is the proportion of DEG *versus* genes not differentially expressed. Each point was colored to represent the adjusted p-value using the Benjamini-Hochberg method.

box O and tumor necrosis factor (TNF) pathways. Gene Set Enrichment Analysis (GSEA) assigned directional change to each pathway and ranked genes were then mapped against the Gene Ontology (GO) database. Encouragingly, pathways involved in antigen presentation and antigen binding were all suppressed (**Figure 2D**), consistent with literature demonstrating that tolDC negatively regulate the immune response.

TolDC Gene Set Validation

Based on the initial discovery set, we identified 3 appropriate gene sets for validation (**Table 4**), annotating each gene by the expected enrichment direction (**Figure 3A**). Our gene signature fit data from TLR- and interluekin-10-generated tolDC, although GM-CSF-generated tolDC performed poorly in this validation step. We conducted further validation of our tolDC gene set using data

TABLE 2A | Top 10 differentially upregulated genes in tolDC.

Dataset (GEO ID)	Method of Generation	Number of DE Genes	Top 10 DE Gene (Upregulated)
GSE13762	Vitamin D	77	SHE, CYP24A1, DRAM1, ST6GAL1, CD2AP, NRIP1, AOAH, G0S2, C20orf197, MIR3945HG
GSE23371	Interleukin 10 & Dexamethasone	140	RNASE1, S100A8, CD163, SELENOP, CD14, SLC18B1, LINC01094, MERTK, C1QB, ADAMDEC1
GSE56017	Dexamethasone	218	TNFAIP6, CCL20, C17orf58, NFKBIA, KYNU, PNRC1, SOD2, TNFAIP3, CYTIP, STK26
GSE117946	Interleukin 10	68	FAM20A, IGF2BP3, FPR1, HIVEP2, CR1, FCGR3A, C1S, CD163, IL7, TGFA
GSE52894	Dexamethasone & Vitamin D	196	C20orf197, UBASH3B, SLC37A2, CA2, COQ2, FBP1, SIGLEC6, LRRC8A, ST6GAL1, ATP5PF

from (19) (**Table 4**) who compared transcriptomic signatures from tolDC derived from 3 different treatments (vitamin D, dexamethasone or rapamycin). Rapamycin-derived tolDC demonstrated a significant genomic deviation from our gene signature (**Figure 3B**).

Alternatively-Activated tolDC

Propagated tolDC that are "alternatively activated" (AADC) by exposure to an inflammatory stimulus, typically LPS, also demonstrate robust regulatory properties that protect against graft-*versus*-host disease (33, 34). AADC have shown greater efficacy in controlling inflammatory immune responses *in vivo* (35) compared to a more modest effect from IL-10-conditioned tolDC (36). We initially interrogated three datasets that compared gene expression between AADC and tolDC, although these demonstrated different DEG (**Figure 4A**, **Table 5**). Analysis determined 39 DEG that were enriched in AADC compared to tolDC (**Table 4** and **Figure 4B**), and we mapped these to GEA pathways (**Figures 4C, D**).

DC Signatures in Tissue

The liver is unique amongst solid organs in its capacity to modulate local and systemic tolerance. This is contributed to by the presence of unconventional antigen presenting cells (liver sinusoidal endothelial cells, Kupffer cells) (37), altered T cell proportions (particular γδ subsets) (38, 39), and an increased ratio of DC to parenchymal cells (2-5 times higher in liver compared to other organs) (40). Importantly, liver-resident DC demonstrate features most consistent with a tolerogenic phenotype and function, with low endocytic capacity, decreased MHC expression, limited T cell allostimulation and high IL-10 production (41–43). Using scRNAseq samples from healthy human liver which has been clustered by cell type (**Figure 5A**, **Supplementary Figure 2A**), we then demonstrated that upregulated genes within the tolDC signature was enriched in areas which mapped to DC/monocyte/macrophage lineage within the liver (**Figures 5B–D**). Downregulated genes were not overexpressed in any cell type (**Supplementary Figure 2B**). We also interrogated whether our tolDC signature was overexpressed in the kidney (which has significantly lower tolerogenic capacity) and/or PBMC. We were able to demonstrate that our gene signature was not enriched in either compared to liver (**Figure 5E**), although an analysis of housekeeping genes (44) was not significantly different (**Supplementary Figure 2C**).

The Relevance of DC Gene Signatures *In Vivo*

DC are rare populations within the peripheral blood (45), but reside at greater frequency within tissue interstitial compartments in an immature state, and sample the environment in organs exposed to potential (neo-)antigens in lung (46, 47), kidney (48), and skin (49, 50). The potential for exogenous stimuli to initiate DC activation suggests that the mature DC gene signature might be enriched in tissue-specific DC subsets *in vivo*. A total of 64 genes were significantly differentially expressed between the mature and immature DC, and the top 52 genes were heat-mapped (**Figure 6A**). The enrichment analysis yielded pathways relevant to cell inflammation and infection (**Figures 6B, C**). Mature DC are well-defined in the literature, and the correlation with an inflammatory gene signature demonstrates the reliability of our pipeline to resolve genes according to DC phenotype, as well as supporting the current hypothesis that DC are influenced by the surrounding environment (3).

To further demonstrate the physiological relevance of our DC gene signatures, we used a dataset identifying 6 myeloid cell subsets (31), demonstrating that our mature DC gene set correlated with the appropriate (mature) DC subset identified

TABLE 2B | Top 10 differentially downregulated genes in tolDC.

Dataset (GEO ID)	Method of Generation	Number of DE Genes	Top 10 DE Gene (Downregulated)
GSE13762	Vitamin D	77	IRF4, IER3, TRIM36, SPIN4, HCAR2, MMP12, CH25H, WFDC21P, CD1e, NUCB2
GSE23371	Interleukin 10 & Dexamethasone	140	MMP12, ALOX15, CDH1, CH25H, APOL4, LAMP3, CCL17, MAFF, ACOT7, SOCS1
GSE56017	Dexamethasone	218	RGS18, TSPAN32, NRGN, NCAPH, KIAA0930, C11orf45, CD1a, ACOX2, LPCAT4, DDIAS
GSE117946	Interleukin 10	68	SCRN1, B3GNT5, PLPP1, CD1c, HCAR3, TIFAB, ATP1B1, MAP4K1, CDH1, FABP4
GSE52894	Dexamethasone & Vitamin D	196	SLC47A1, CD1c, ESYT1, RGS18, ABCA6, DHRS2, CLIP2, HLA-DMB, DOCK10, CALCRL

TABLE 3 | Summary of differentially expressed genes in tolDC.

Upregulated Genes	Downregulated Genes
DRAM1, NRIP1, CEBPB, SMPDL3A, NOD2, CD14, PAPSS2, ST3GAL1, SEMA6B, CD300LF, ACSL1, TREM1, NINJ1, NCF1C, RGS18, TSPAN14, MS4A4A, CD93, NCOA4, BRD8, C1QA, GK, C5AR1, EPB41L3	IRF4, TRIM36, MTCL1, HCAR2, MMP12, KCTD6, ZFP69, PP1R16A, CD1A, CD1E, CD1B, CD1C, IL1RAP, ESYT1, CALCRL, NCAPH, BCAR3, PEA15, FCER1A, SCRN1, GALNT12, NDRG2, ISYNA1, SLC27A3, NRGN, KIAA0100, VCL, CDH1, C1orf115

in vivo (**Figure 6D**). This also shows our approach to identifying a cell-specific gene signature on microarray platforms could be successfully applied to RNAseq data. Interestingly, the tolerogenic and mature DC gene sets could also be applied to distinct immune cell subsets within peripheral blood (32), with the latter enriched in myeloid DC (mDC) and monocytes (**Figure 6E**). We applied our mature DC signature to liver, kidney and PBMC scRNAseq samples, demonstrating significantly lower expression in liver (**Figure 6F**). Kidney-resident DC and PBMC showed an enhanced mature DC signal compared to tolDC (**Figure 6G**). We also interrogated a recent dataset comparing the expression profiles of mononuclear phagocytes (MNP) isolated from epidermal and dermal tissue (30). The expression of our mature, but not tolerogenic, DC signature was significantly higher in recognized DC subsets (**Figure 6H** and **Supplementary Figures 3A, B**).

DISCUSSION

Here we derive novel, distinct genetic signatures for both tolDC and mature DC. Both gene sets align with known biological differences in phenotype and function, and can be used to identify physiological DC subsets *in vivo*. Most interesting was the mapping of the tolDC signature to liver DC. Our analysis also demonstrated that tolDC and immature DC are distinct subsets, despite current paradigms suggesting overlap of several features (51), and these data support the notion that tolDC indeed derive from specific transcriptional programming.

We identified several genes critical to tolDC function. Several compartments of the CD1 glycoprotein complex were downregulated in the tolDC gene set. CD1 is a cell surface protein that is involved in presentation of lipid-based antigens to T-cells and natural killer cells that subsequently mediate adaptive immunity (52, 53). CD1 autoreactive T-cells, particularly CD1a and CD1c, are abundant among circulating T-cells from healthy human adults and neonates (54) and are associated with a variety of diseases. The plasticity of CD1 antigen presentation highlights evolved mechanisms that regulate the self/non-self cellular lipid environment presented to T-cells. With CD1a-c expression decreased in the tolDC we can speculate defective T cell stimulation ability due to altered antigen processing and presentation (55). This finding has also been replicated in tissue-resident CD103+ conventional DC which were less effective in antigen cross-presentation with accumulated lipid bodies (56).

CD14, a known monocyte cell-surface marker in blood, is expressed by tissue-based macrophages, and was significantly upregulated in tolDC. CD14 has several functions on the surface of monocytes, ranging from metabolism to pathogen-associated-molecular pattern (PAMP) identification in the innate immune response (57). CD14 binds to extracellular LPS and acts as a secondary receptor to TLR4 in facilitating a subsequent immune response (58). However, recent data has demonstrated that DC subsets expressing CD14 impeded T-cell proliferation (59). Interestingly, CD14 and CD1a kinetics are replicated in human monocyte-derived DC whose maturation capacity are limited by co-culture with immune complexes (60).

The global gene expression profile of tolDC identified prominent enrichment of the mitogen-associated protein kinase (MAPK) pathway. This finding is in keeping with reports that MAPK (specifically p38) inhibition promotes an immunogenic DC phenotype (61) and augments effector T cell responses (62). Cytoskeletal pathway changes (specifically related to actin filaments) were suppressed, a process that is fundamental to plasma membrane internalization for endocytosis and vesicle

TABLE 4 | Identified publicly available gene datasets including immature, tolerogenic and mature DC for tolDC gene set validation.

Dataset ID	Platform ID	References	Sample Proportions	Agent Used to Induce the tolDC Phenotype
GSE104438	GPL14550	(26)	4 x Macrophage, 4 x imDC 4 x tolDC	Low dose GM-CSF
GSE98480	GPL10558	(27)	3 x imDC 3 x imDC + LPS 3 x tolDC 3 x imDC + Poly I:C	Toll like receptor 7/8 ligand (R848)
GSE92852	GPL18460	(28)	3 x imDC 3 x imDC + LPS 3x tolDC 3 x tolDC + LPS	Interleukin-10
E-MTAB-6937 (ArrayDatabase)	–	(19)	5 x imDC 5 x imDC + LPS 5 x rapa-tolDC 5 x dexa-tolDC 5 x vitD3-tolDC	Rapamycin Dexamethasone Vitamin D

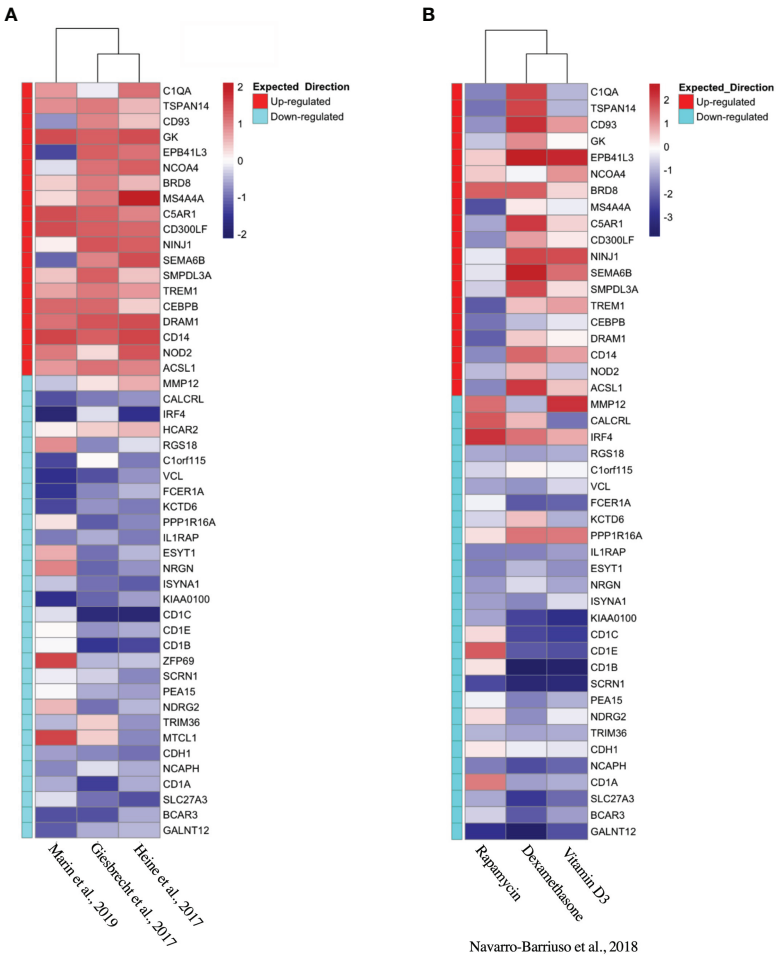

FIGURE 3 | Validation of tolDC transcriptome. Heatmap representation of upregulated and downregulated genes from the tolDC discovery gene set compared to expression in **(A)** GEO-derived or **(B)** ArrayDatabase validation gene set.

transportation required for antigen processing and cell surface presentation (63, 64).

Our tolDC gene set was validated in datasets from publications generating tolerogenic human DC using a variety of pharmacological agents (TLR ligands, IL-10, vitamin D and dexamethasone). TolDC propagated using GM-CSF (26) or rapamycin (19) demonstrated noticeably different transcriptomes, in keeping with known phenotypic and functional differences (although direct *in vitro* comparisons were not consistently reported). GM-CSF alone is not commonly used *in vitro* for this purpose, and has been shown to produce tolDC that are distinct from the established literature, including greater plasticity (65) and metabolic changes that drive T cell inhibition (26). Rapamycin-induced tolDC also diverge from other tolDC, producing higher bioactive IL-12 and lower IL-10 levels (66), in addition to strikingly discrepant findings of mTOR inhibition on DC function that demonstrate activation (67, 68) or inhibition (69, 70).

Alternatively-activated DC (AADC), tolerogenic DC activated by inflammatory stimuli, are effective in inducing anergic and regulatory T cell responses (34) that protects against lethal graft-*versus*-host-disease in pre-clinical models (33). Only 3 comparative datasets were available for analysis and did not demonstrate homogeneity between DEG from AADC and tolDC. Gene enrichment analysis demonstrated increased virus and stress responsiveness, as well as cytokine-signaling/inflammatory pathways, with concurrent downregulation of mitochondrial function. Metabolic plasticity, including enhanced catabolism, has been correlated with DC function, and our findings correlated with previous work demonstrating decreased oxidative phosphorylation capacity with LPS-stimulated tolDC (24).

TolDC are artificially generated *in vitro*, and therefore not wholly representative of DC found physiologically. However, natural and induced DC with tolerogenic capacity (71) are crucial for homeostatic function, particularly in tissues exposed to environmental stimuli. The liver is considered the most tolerogenic organ, and our tolDC gene signature was overrepresented in four integrated scRNAseq datasets of healthy human liver, clustering with liver-resident DC (with overlap seen in the macrophage/monocyte population). DC and macrophages are interrelated, derive from common lineages, and are often phenotypically and functionally indistinguishable (51).

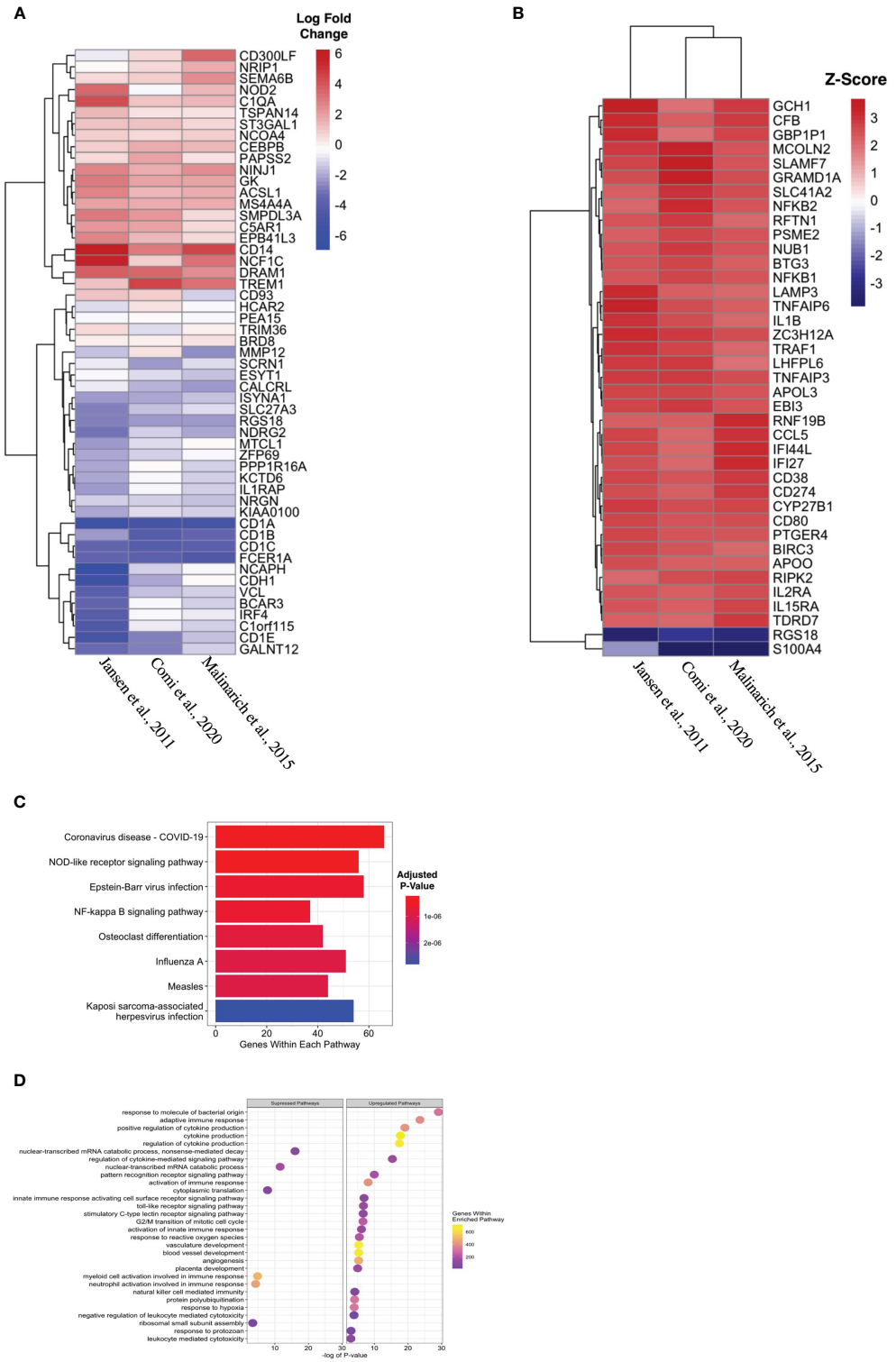

FIGURE 4 | Identifying transcriptomic differences between subtypes of tolDC. **(A)** Heatmap representation of the top 39 DEG by AADC. **(B)** Fold change difference in expression of genes in AADC compared to tolDC. **(C)** KEGG and **(D)** Gene Set Enrichment analyses.

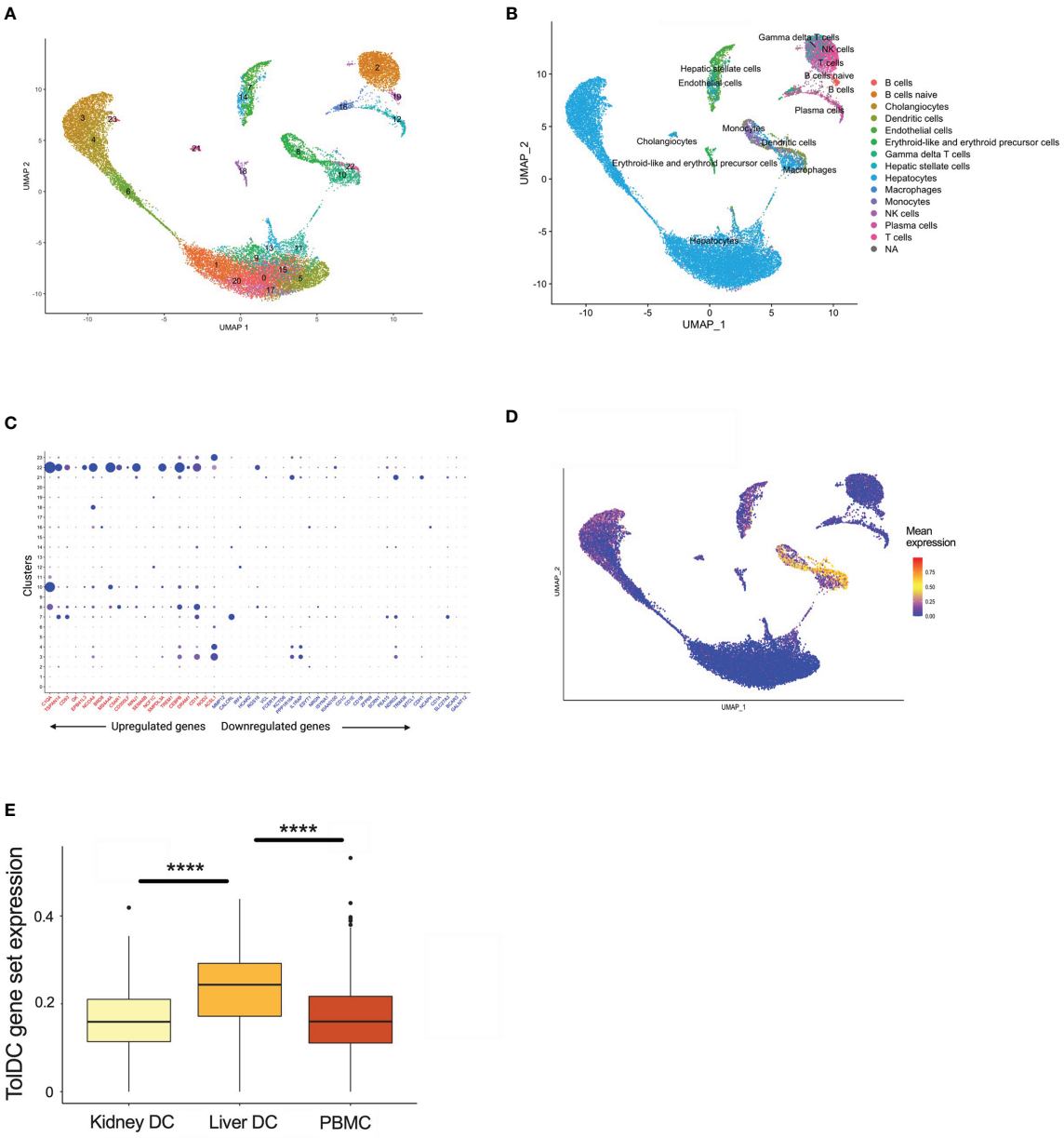

FIGURE 5 | TolDC gene set is overexpressed in liver-resident DC. **(A)** UMAP plot displaying the clustering of harmony integrated scRNAseq samples. **(B)** UMAP plot of liver datasets annotated by cell type. **(C)** Dot plot displaying up- and down-regulated tolDC gene expression markers enriched within cell clusters. **(D)** UMAP plot demonstrating a joint density analysis of upregulated genes from the tolDC gene set. **(E)** Boxplot displaying the expression of the tolDC gene set across tissue-resident and circulating DC. ****$p < 0.0001$.

Hepatic DC are distinct from other tissue-based DC (37, 72), abundantly secreting immunosuppressive cytokines (41, 73) that dictate immunoregulatory properties. We mapped the tolDC gene set to scRNAseq samples of healthy (and more immunogenic) kidney as a comparator, but the signature was not overexpressed, in keeping with clinical and experimental data that support organ-specific differences in allograft acceptance (74).

Our pipeline generating a tolDC transcriptomic signature was applied to developing a gene set relevant to mature DC. Genes deemed significant to mature DC were strongly implicated in the inflammatory response and, using the KEGG database, mapped to TNF-α and NF-kB signaling pathways. NF-κB is a central mediator of pro-inflammatory gene induction and functions in both innate and adaptive immune cells, and central for DC maturation (75). We were able to demonstrate that our mature DC gene signature was enriched in CD1c+ mature DC rather than CLEC9A+ immature DC. These findings, while not novel, speak to the validity of our methods in characterizing DC

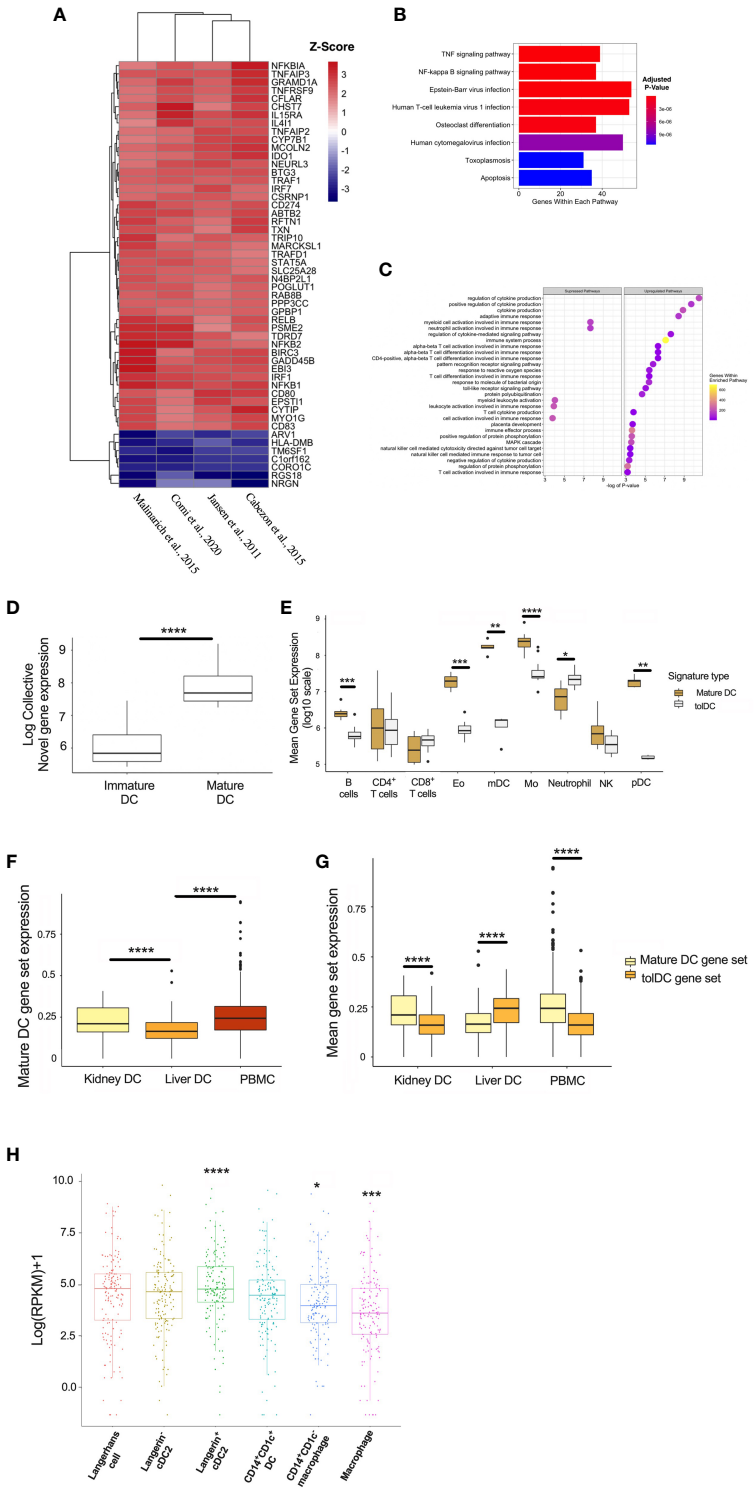

FIGURE 6 | Generating a mature DC transcriptome. **(A)** Heatmap representation of the top 52 DEG within the mature DC phenotype. **(B)** KEGG and **(C)** Gene Set Enrichment Analyses. **(D)** Mature DC gene-set expression in myeloid cell subsets isolated from epithelial tissues. **(E)** Comparison of tolDC and mature DC gene signatures in peripheral blood immune cell subsets. **(F)** Boxplot displaying the expression of genes critical to mature DC across DC in liver, kidney and PBMC. **(G)** Comparison of tolDC and mature DC gene set expression in liver, kidney and PBMC. **(H)** Mononuclear phagocytes from epithelial and subepithelial tissues were isolated and classified as DC or macrophage. The average expression of the mature DC gene signature was plotted between cells. A two-sample t-test was performed to determine statistically significant differences in base mean expression of the mature DC gene set across MNP. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

TABLE 5 | Summary of differentially expressed genes in AADC.

Upregulated Genes	Downregulated Genes
BTG3, NF-KB1, NF-KB2, RFTN1, SLC41A2, SLAMF7, GRAMD1A, LHFPL6, NDP, MCOLN2, PSME2, IFI27, IFI44L, RNF19B, GCH1, GBP1P1, APOO, CCL5, CD274, CYB27B1, G0S2, CD38, CD80, CFB, TNFAIP6, ZC3H12A, TNFAIP3, APOL3, NUB1, LAMP3, IL-1B, TRAF1, EBI3, PTGER4, BIRC3, RIPK2, IL2RA, IL15RA, TDRD7	RGS18, S100A4

phenotype using gene expression datasets, and demonstrate that our signature could be applied to physiological DC *in vivo*.

This paper further highlights the need for further -omic studies to identify a consensus gene expression profile, including distinct signaling pathways, that can confirm tolDC function and stability *in vivo*. Despite the reported safety of tolDC in early-phase human trials (17), and known efficacy in large animal models (76), potential variability in clinical grade tolDC preparations remains a concern for translational purposes. The advent of standardized tolDC manufacturing through Focus and Accelerate Cell-based Tolerance-inducing Therapies (77) aims to minimize variations in approach and is a key step towards a standardized tolDC production for pre-clinical studies and clinical trials. Understanding the genomic processes behind the functional properties of DC and identification of molecular targets of immunomodulation provide potential opportunities for intervention to silence unwanted immune responses.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analysed in this study. This data can be found here: GSE13762, GSE23371, GSE56017, GSE117946, GSE52894, GSE104438, GSE98480, GSE92852, ArrayExpress database E-MTAB-6937.

AUTHOR CONTRIBUTIONS

NR and EP led the study, designed experiments, and wrote the manuscript. HR, JL, and EP analysed the data. All authors contributed to experimental design and drafting the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.733231/full#supplementary-material

Supplementary Figure 1A | Principal component analysis (PCA) from tolDC discovery and validation datasets. PCA plots characterizing the change in gene expression profiles between immature DC (red), mature DC (green), tolDC (blue), or alternatively-activated tolerogenic DC (AADC, purple).

Supplementary Figure 1B | Heatmap demonstrating the correlation between samples. Gene signatures from immature DC (blue), mature DC (red), tolDC (purple), and AADC (green) from relevant datasets. Both the horizontal and vertical axis were clustered using the same hierarchical clustering algorithm. Each square on the heatmap is the value of Pearson's correlation coefficient between the two sample, and values are assigned a color.

Supplementary Figure 2 | TolDC gene signature expression in human kidney tissue. **(A)** UMAP plot of human liver datasets integrated using the harmony method. Each dataset was annotated using the accessible code on the Panglao database. **(B)** UMAP plot demonstrating a joint density analysis of downregulated genes from the tolDC gene set. **(C)** Boxplot displaying the expression of housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex subunit A (SDHA) and peptidylprolyl isomerase A (PPIA) across liver and kidney DC, and PBMC.

Supplementaary Figure 3 | TolDC gene set expression in human mononuclear phagocytes. Mononuclear phagocytes were isolated from epithelial and sub-epithelial tissues. The average expression of the tolDC gene signature was plotted between cells. The average expression of the tolDC gene signature was plotted between cells. A two-sample t-test was performed to determine differences in base mean expression of the tolDC gene set across MNP. **(B)** Boxplot displaying differences between tolDC and mature DC transcriptomic signatures within each MNP subset. $^{**}p < 0.01$, $^{***}p < 0.001$.

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Unraveling the Effects of a Talimogene Laherparepvec (T-VEC)-Induced Tumor Oncolysate on Myeloid Dendritic Cells

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T-VEC, a HSV-1 derived oncolytic virus, is approved for the treatment of advanced melanoma. The mechanisms that underly the systemic anti-tumor effect that is seen following intratumoral injection have not yet been studied but are likely to be mediated by myeloid dendritic cells (myDC) that initiate an adaptive immune response. In this study we could demonstrate that T-VEC is non-toxic for human myDC. T-VEC and a T-VEC oncolysate of melanoma cell lines were able to mature human myDC. myDC were able to take up lysed melanoma cells and cross-present melanoma-derived tumor antigens to antigen-specific T cells. Our results support the possible role of myDC as mediators of an adaptive anti-tumor effect and intratumoral co-administration of T-VEC plus autologous myDC could be a complementary treatment option. A clinical trial that investigates this hypothesis is currently ongoing.

Keywords: melanoma, myeloid dendritic cell, BDCA-1, BDCA-3, Talimogene laherparepvec, cell therapy, immunotherapy

INTRODUCTION

Dendritic cells (DC) are essential for the initiation of an adaptive immune response. They act as a bridge between the innate and the adaptive immune system using their unique capabilities to activate naive lymphocytes by capturing, processing and presenting antigens. DC are generally characterized by a high expression of major histocompatibility complex (MHC) class II molecules and CD11c, but are extremely heterogeneous both in phenotype and function. All human DC arise from a $CD34^+$ hematopoietic precursor, and differentiate subsequently into monocyte, macrophage and DC precursor cells (MDP), common DC precursor cells (CDP) and then into either plasmacytoid DC (pDC) or preclassical DC (pre-cDC). Pre-cDC can evolve into two types of classical or conventional DC (cDC), also called myeloid DC (myDC): cDC1 and cDC2. cDC1 are

characterized by expression of CD141/BDCA-3, XCR1, CLEC9A and the BATF3 transcription factor. cDC2 are more heterogenous. They are characterized by expression of CD1c, BDCA-1 and SIRPα and are the most abundant type of cDC in the blood circulation (1). Recently, this classification was refined based on single cell RNA sequencing data that identified in total six different types of DC. Within this novel classification, cDC1 correspond to the DC1 subclass. cDC2 are subclassified in DC2 (MHC class II-like) and DC3 ($CD14^+$ monocyte-like). Both cell types arise from a specific circulating and dividing cDC progenitor cell (2). For therapeutic use, another type of DC is frequently used: monocyte-derived DC (moDC). These are generated *in vitro* by stimulating $CD34^+$ precursors or $CD14^+$ monocytes with GM-CSF and TNF-alpha ($CD34^+$) or IL-4 (monocytes). Not much is known about the *in vivo* differentiation of monocytes into moDC (3).

However, recent observations indicate that moDC might not be the best suited DC type to use for therapeutic purposes. It has been demonstrated that moDC have a decreased migratory capacity, present with a more exhausted phenotype (decreased cytokine secretion and T cell stimulatory capacity) and are generally less potent. Our research focuses on cDC1 and cDC2 as these cell types have been shown to be able to re-invigorate the cancer immunity cycle and are key to the cross-presentation of tumor antigens (4, 5). It has been shown that these cells are deficient in several cancer types, and deficiency of these cell types is correlated with a worse outcome (6). However, it has long been impossible to isolate these cells in sufficient numbers to allow therapeutic use, due to the absence of antibodies of sufficient quality (affinity and specificity) to perform their isolation. cDC1 ($CD141^+$) are only present in very limited quantities in the blood (one-tenth the frequency of cDC2) (7). cDC1 have been found to be of critical importance in 'relicensing' the anti-tumor activity of $CD8^+$ cytotoxic T cells in the tumor micro-environment. Recently, it has been shown that cDC1 are important for early priming of $CD4^+$ helper T cells even though this was long hypothesized to be the specialization of cDC2 (8–10). Only limited (clinical) data is currently available regarding the therapeutic use of these cell types (11).

DC need to successfully perform several functions to trigger an effective adaptive immune response. First, they should take up tumor-associated antigens, then (concurrently) they need to be stimulated in order to become activated. After activation, mature DC must migrate to the lymph node area where they present tumor antigens to antigen-specific T cells. Adequate stimulation must be present in the tumor environment for DC to become mature. Upon induction of cell death, cells release a variety of molecules in their environment depending on both cell type and type of cell death. These cell death-induced mediators can be detected by various pattern recognition receptors (PRR). The most widely known PRR are the so-called Toll-like receptors (TLR) which are differentially expressed on the different types of myDC. It has been shown that $CD1c^+$ myDC express all TLR except for TLR9. In contrast, $CD141^+$ myDC exhibit a restricted pattern of TLR expression with high expression of TLR3 and TLR10, intermediate expression of TLR1, -2, -6 and -8, and no expression of TLR4, -5, -7 and -9. TLR9 expression is mainly restricted to pDC. However, despite the absence of TLR9 receptors, $CD141^+$ cells have nevertheless shown to produce IL-12 in response to TLR9 agonists pointing to a yet unidentified receptor type (12).

Talimogene laherparepvec (T-VEC, Imlygic®, Amgen) is a herpes simplex virus-1 (HSV-1) derived oncolytic virus (OV) that has been approved by the Food and Drug Administration (FDA) in 2015 for the treatment of local and locally advanced cutaneous melanoma (13, 14). T-VEC selectively replicates in tumor cells and improves the immune response by inducing granulocyte-macrophage colony stimulating factor (GM-CSF) secretion by infected cells. In addition, it has been shown that T-VEC induces immunogenic cell death in melanoma cell lines with the associated release of damage-associated molecular patterns (DAMPs), as measured by release of high mobility group box-1 (HMGB-1), adenosine triphosphate (ATP) and ecto-calreticulin (CRT). HMGB-1 release results in stimulation of TLR2, TLR4 and RAGE receptors (15, 16). Release of ATP interacts with DC through the P2X7 and P2Y2 receptor in order to attract immune cells and act as a 'find-me' signal (17). CRT functions as an 'eat-me' signal by activating the CD91 (LRP1) receptor and strengthens the immune response by releasing pro-inflammatory cytokines leading to Th17 priming (18).

The mechanisms by which intratumorally injected T-VEC generates a protective systemic anti-tumor effect have not been elucidated. Presumably intratumoral T-VEC administration reactivates a cancer immunity cycle by lysing tumor cells and providing viral elements that activate antigen presenting cells. These antigen presenting cells can re-initiate an adaptive immune response. In this study we have taken advantage of the availability of human myDC obtained within the context of a clinical trial to study their interaction with T-VEC.

MATERIALS AND METHODS

Cell lines and Reagents

The human-derived melanoma cell lines 624-mel and 938-mel were a kind gift from prof. S. Topalian (Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine) to prof. Aerts. Cells were cultured in RPMI-1640 (Life Technologies) supplemented with 10% FBS (Fetal Bovine Serum, Biochrome), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies) and 100 µg/mL streptomycin (Life Technologies) at 37°C, 5% CO_2.

Talimogene laherparepvec (T-VEC, Amgen) was stored at -80°C and thawed for use when applicable.

mRNA encoding for the α and β chain respectively of the NY-ESO-1 and gp100 T cell receptor (TCR) was produced by Prof. Breckpot's group at the Laboratory of Molecular and Cellular Therapy (LMCT, VUB). Briefly, gBlocks™ (Integrated DNA Technologies, IDT) encoding for the α and β chain sequence of the T cell receptor recognizing the NY-ESO-1 peptide (SLLMWITQV) and gp100 peptide (YLEPGPVTA) were cloned into the *in-house* developed plasmid LMCT (pLMCT).

pLMCT was linearized (NcoI/XhoI, Thermo Fisher Scientific) and each TCR chain sequence was cloned into the background vector *via* Gibson assembly reaction (New England Bio Labs, NEB). Cloned plasmids were screened *via* enzymatic restriction digestion and sequences were verified (Eurofins Genomics). Plasmids were prepared and purified according to Qiagen protocol (Qiagen-Plasmid Midi Prep®, Qiagen/Filter service). Purified TCR plasmids were linearized with BfuA1 (NEB) restriction enzyme prior mRNA *in vitro* transcription. T7 RNA polymerase (Thermo Fisher Scientific) together with co-transcriptional capping reagent CleanCap® AG (TriLink Biotechnologies) were used in the iVT reaction mix. The resulting mRNA was purified *via* NaCl/EtOH precipitation and resuspended in water for injection at 1µg/µL final concentration. mRNA integrity and identity were verified with Agilent Bioanalyzer RNA 6000 Nano Kit®.

The NY-ESO-1 peptide (SLLMWITQV) was purchased from Fisher Scientific. The gp100 peptide (YLEPGPVTA) was a kind gift from prof. K. Breckpot.

Incucyte® Proliferation Assay

Tumor cells were plated in flat-bottom 96-well plates at a density of 1 x 10^4 cells per well and left to adhere overnight at 37°C, 5% CO_2 in the Incucyte® Zoom instrument (Sartorius). The following day, T-VEC was added at the indicated multiplicity of infection (MOI). All conditions were tested in triplicate. Cell growth was monitored continuously with a 10x objective using 2h intervals. Cell proliferation was assessed by analyzing the occupied area (% confluence) over time using the Incucyte® analysis software (Sartorius).

GM-CSF Production

Tumor cells were plated in flat-bottom 96-well plates at a density of 1 x 10^4 cells per well and left to adhere overnight at 37°C, 5% CO_2. The following day, T-VEC was added at the indicated multiplicity of infection (MOI). All conditions were tested in triplicate. Supernatant was harvested after 24, 48 and 72h and stored at -20°C. GM-CSF content in these supernatants was assessed using an ELISA (Biolegend), according to manufacturer's instructions. Optical density was read at 450 nm and 570 nm using an xMark™ absorbance spectrophotometer (Bio-Rad Laboratories) and GM-CSF concentrations were calculated using Microplate Manager software (Bio-Rad Laboratories).

Myeloid Dendritic Cell Isolation

Isolated myDC were obtained from patients included in various clinical trials at UZ Brussel from whom excess myDC were available for translational research. Those studies have been conducted in accordance with the Declaration of Helsinki and were approved by the ethics committee of the UZ Brussel. The patients provided written informed consent to use cells that were not used for treatment for research purposes. Briefly, patients underwent a leukapheresis and next, $CD14^+$ and $CD19^+$ cells were depleted using CliniMACS® CD14 reagent and CliniMACS® CD19 reagent, followed by positive selection of $CD1c\ (BDCA\text{-}1)^+$ and $CD141\ (BDCA\text{-}3)^+$ myDC using CliniMACS® CD1c (BDCA-1)-biotin, CliniMACS® Anti-Biotin Reagent and CliniMACS® CD141 (BDCA-3) Microbeads (all Miltenyi Biotec) on the immunomagnetic CliniMACS Prodigy® system (Miltenyi Biotec). Cells were cryopreserved in 7.5% DMSO – 8.25% human albumin solution and stored in the vapor phase of liquid nitrogen. The purity of the isolated $BDCA\text{-}1^+/BDCA\text{-}3^+$ cell product was analyzed by flow cytometry. 5 x 10^5 cells were stained with CD14-FITC (Biolegend, clone HCD14), CD45-PE (Miltenyi Biotec, clone REA747), 7-AAD (Thermo Fisher Scientific), CD141-PE/Cy7 (Invitrogen, clone JAA17), CD123-APC-Vio770 (Miltenyi Biotec, clone REA918), FcER-Vioblue (Miltenyi Biotec, clone CRA1), CD11c-Alexa Fluor 700 (BD Biosciences, clone B-ly6), CD1c-Brilliant Violet 510 (BD Biosciences, clone F10/21A3), CD15-APC (Invitrogen, clone MMA) for 20 minutes at 4°C. After washing, cells were resuspended in PBS/0.5%BSA and acquired on a BD LSR Fortessa instrument (BD Biosciences). Data analysis was performed using FlowJo and FCS Express 7 software. Purity was evaluated as follows: cells were gated based on FSC/SSC characteristics and subsequently on viable $7\text{-}AAD^-$, $CD45^+$ cells. On this gate, CD14 and CD15 expressing cells were excluded and then we identified $CD11c^-\ CD123^+$ pDC and $CD11c^+\ CD123^-$ myDC. On the myDC gate, we then identified $BDCA\text{-}1^+$ DC as $CD1c^+\ CD141^-\ Fc\varepsilon R^+$ cells and $BDCA\text{-}3^+$ DC as $CD1c^-\ CD141^+$ cells.

Effect of T-VEC or Supernatant From T-VEC-Treated Melanoma Cells on myDC

Purified $BDCA\text{-}1^+/BDCA\text{-}3^+$ myDC were cultured at 1.5-2 x 10^5 cells per 96-well (ultra-low attachment) in 200 µL X-VIVO-15 (Lonza, Belgium) supplemented with L-glutamine, penicillin-streptomycin and sodium pyruvate (DC medium) in the presence of 1000 U/mL GM-CSF (Miltenyi Biotec). T-VEC was added at a multiplicity of infection (MOI) of 1 and incubated for 24h at 37°C, 5% CO_2. As a control, heat-inactivated T-VEC was also used (15 min at 65°C, followed by 1 min at 100°C). The combination of 20 µg/mL poly(I:C) and 4 µg/mL R848 was used as a positive control. Alternatively, supernatant of melanoma cells (624-mel or 938-mel) treated with T-VEC at MOI 1 for 24h or 48h was added to the DC and incubated for 24h at 37°C, 5% CO_2. The supernatant of the cultures was harvested and stored at -20°C for cytokine analysis and the phenotype of the $BDCA\text{-}1^+/BDCA\text{-}3^+$ myDC was analyzed by flow cytometry.

Flow Cytometric Analysis

For analysis of the phenotype of $BDCA\text{-}1^+/BDCA\text{-}3^+$ myDC, cells were stained with CD11c-Alexa Fluor 700 (BD Biosciences, clone B-ly6), CD1c-Brilliant Violet 510 (BD Biosciences, clone F10/21A3), CD141-PE/Cy7 (Invitrogen, clone JAA17), CD274-PE-CF594 (BD Biosciences clone MIH1), CD86-Brilliant Violet 421 (BD Biosciences, clone 2331 (FUN-1)), CD83-PE (BD Biosciences, clone HB15e), CD40-APC (BD Biosciences, clone 5C3), CD80-PE/Cy5 (BD Biosciences, clone L307.4), HLA-ABC-FITC (BD Biosciences, clone G46-2.6), Zombie Yellow (Biolegend) for 20 minutes at 4°C. After washing, cells were

resuspended in PBS/0.5%BSA and acquired on a BD LSR Fortessa instrument. Data analysis was performed using FlowJo software. The gating strategy was as follows: cells were first gated on FSC/SSC characteristics, followed by gating on single cells. Next, dead cells were excluded and subsequently we gated on the $CD11c^+$ population. On this gate, $CD1c^+$ $CD141^-$ cells were identified as BDCA-1^+ myDC and $CD1c^-$ $CD141^+$ cells as BDCA-3^+ myDC. Subsequently, we evaluated for each myDC subtype the expression of HLA-ABC, CD83, CD274/PD-L1, CD80, CD40 and CD86.

Cytokine Detection

The supernatants of myDC treated with T-VEC or with supernatant of melanoma cells treated with T-VEC was analyzed for its content of the following cytokines: IL-10, IL-12p70, TNF-α, IFN-α-2a and IFN-λ1 using a human multiplex U-plex assay (MesoScale Diagnostics) according to manufacturer's instructions and analyzed using a MESO QuickPlex SQ 120 instrument (MesoScale Diagnostics). Data were analyzed using the MSD Discovery Workbench software.

Phagocytosis Assay

In order to measure the uptake of dying melanoma cells by BDCA-1^+/BDCA-3^+ myDC, we have set-up a co-culture assay between pHrodo™-labeled tumor cells and BDCA-1^+/BDCA-3^+ myDC.

To this end, 938-mel cells were plated at 2.5 x 10^5 cells per 12-well and left to adhere overnight. The next day, T-VEC was added to these cells at an MOI of 1. After 24h incubation with T-VEC, treated cells were harvested and subsequently labeled with the pHrodo™ Deep Red Mammalian and Bacterial Cell Labeling Kit (Thermo Fisher Scientific), according to manufacturer's instructions. Next, pHrodo™-labeled, T-VEC-treated 938-mel cells were co-cultured with BDCA-1^+/BDCA-3^+ myDC at different ratio's during 2, 4 or 6h. At these time points cells were harvested and stained for CD11c-Alexa Fluor 700 (BD Biosciences, clone B-ly6), CD1c-Brilliant Violet 510 (BD Biosciences, clone F10/21A3), CD141-PE/Cy7 (Invitrogen, clone JAA17), CD45-PE (Miltenyi Biotec, clone REA747), CD123-PE/Cy7 (Miltenyi Biotec, clone REA918) and 7-AAD (Thermo Fisher Scientific) for 20 min at 4°C. After washing, cells were resuspended in PBS/0.5%BSA and acquired on a BD LSR Fortessa instrument. The pHrodo™ Deep Red dye was measured in the Cy5 channel. Data were analyzed using FlowJo software using the following gating strategy: cells were first gated based on FSC/SSC characteristics, followed by gating on single cells. Next, we selected the viable, 7-AAD^-, $CD45^+$ cells and then we selected myDC as $CD11c^+$ $CD123^-$ cells. On this gate, BDCA-1^+ DC were identified as $CD1c^+$ $CD141^-$ cells and BDCA-3^+ DC as $CD1c^-$ $CD141^+$ cells. Uptake of pHrodo™-labeled tumor cells was assessed by evaluating the percentage of Cy5-expressing cells in the $CD11c^+$ DC, BDCA-1^+ DC and BDCA-3^+ DC gates respectively.

Antigen Presentation Assay

On day 0, 938-mel cells were plated at 2.5 x 10^5 cells per 12-well and left to adhere overnight. The next day, T-VEC was added to these cells at a MOI of 1. After 24h incubation with T-VEC, treated cells were harvested. On day 2, BDCA-1^+/BDCA-3^+ myDC from an HLA-A2-positive donor were thawed in a 37°C water bath and diluted with PBS/4% human serum. After centrifugation, cells were resuspended in DC medium and counted with trypan blue. BDCA-1^+/BDCA-3^+ myDC were co-cultured with T-VEC-treated 938-mel cells at different ratio's for 24h. BDCA-1^+/BDCA-3^+ myDC alone were used as controls. On day 2, autologous T cells were thawed, counted with trypan blue and cultured in IMDM (Iscove's Modified Dulbecco's Medium, Life Technologies) containing 1% human serum, penicillin-streptomycin, L-glutamine, sodium pyruvate and non-essential amino acids (T cell medium) at a density of 5 x 10^6 cells/mL in the presence of 250 U/mL IL-2 for 24h. On day 3, T cells were harvested, counted and electroporated with mRNA encoding the α- and β-chain of the TCR for NY-ESO-1 or gp100 (10 µg RNA per 4 x 10^6 T cells, square wave pulse, 500V, 1 pulse, 5 ms, 4 mm cuvette). After electroporation, the cells were transferred to 2 mL T cell medium containing 20 U/mL IL-7 for 1h. Meanwhile, BDCA-1^+/BDCA-3^+ myDC co-cultured with T-VEC-treated 938-mel cells were harvested and counted. Next, these BDCA-1^+/BDCA-3^+ myDC were co-cultured overnight with the autologous TCR-electroporated T cells at a 1:1 ratio in T cell medium. Unmanipulated BDCA-1^+/BDCA-3^+ myDC and BDCA-1^+/BDCA-3^+ myDC loaded with the respective peptides (NY-ESO-1: SLLMWITQV; gp100: YLEPGPVTA), 624-mel cells (NY-ESO-1^+, $gp100^+$, HLA-$A2^+$) and 938-mel cells (NY-ESO-1^+, $gp100^+$, HLA-$A2^-$) were used as controls. On day 4, the supernatant of the DC-T cell co-cultures was harvested and analyzed for IFN-γ content by ELISA (Biolegend), according to manufacturer's instructions. Optical density was read at 450 nm and 570 nm using an xMark™ absorbance spectrophotometer (Bio-Rad Laboratories) and IFNγ concentrations were calculated using Microplate Manager software (Bio-Rad Laboratories).

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism version 9.1.0 software. Live cell imaging data were analyzed using a 1-way ANOVA followed by a Friedman test for multiple comparisons. Phenotypic differences were analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons test. For the phagocytosis assay, a mixed-effects analysis was used, and the antigen presentation assay was analyzed using 1-way ANOVA.

RESULTS

Cell Death Kinetics Induced by Talimogene Laherparepvec (T-VEC) on Melanoma Cell Lines

To study the kinetics of cell death induced by T-VEC, we used the Incucyte® Live Cell Imaging system to continuously monitor the cell growth upon T-VEC treatment. As illustrated in **Figure 1A**, T-VEC treatment induced a time- and dose-dependent inhibition of tumor cell growth for the melanoma cell lines 624-mel and 938-mel. The time upon which cell growth inhibition occurs is MOI-dependent. Both cell lines seem to be almost equally sensitive to T-VEC-mediated growth inhibition.

Since T-VEC encodes the gene encoding for GM-CSF, this cytokine should be produced upon successful infection. In **Figure 1B**, we show the secretion of GM-CSF by T-VEC-treated melanoma cells at 24, 48 and 72h post-infection. Also here, the amount of GM-CSF production increases over time and the levels are MOI-dependent. The differences in GM-CSF secretion between different MOI's are statistically significant as indicated in **Figure 1B**. The levels of GM-CSF produced by both cell lines are comparable.

T-VEC and T-VEC-Induced Oncolysate Activate Myeloid Dendritic Cells (myDC)

We cultured purified BDCA-1^+/BDCA-3^+ myDC in the presence of supernatant of T-VEC-treated melanoma cells and analyzed their phenotype and cytokine secretion pattern. **Figure 2A** shows the purity of the isolated BDCA-1^+/BDCA-3^+ myDC cell products. On average, the BDCA-1^+/BDCA-3^+ myDC cell product contained 68.86 ± 14.42% BDCA-1^+ myDC and 6.71 ± 3.59% BDCA-3^+ myDC, corresponding to a ratio of 10:1 as described before in the blood (7). Upon isolation, these BDCA-1^+/BDCA-3^+ myDC showed an immature phenotype (data not shown). After treatment with supernatant from T-VEC-infected tumor cells, we observed a trend towards an increase in expression of CD80, CD274 (PD-L1) and HLA-ABC on both the BDCA-1^+ and BDCA-3^+ myDC subsets, while levels of CD40, CD83 and CD86 remained constant. **Figure 2B** shows the data for CD80, CD274/PD-L1 and HLA-ABC using supernatant from 938-mel cells treated with T-VEC.

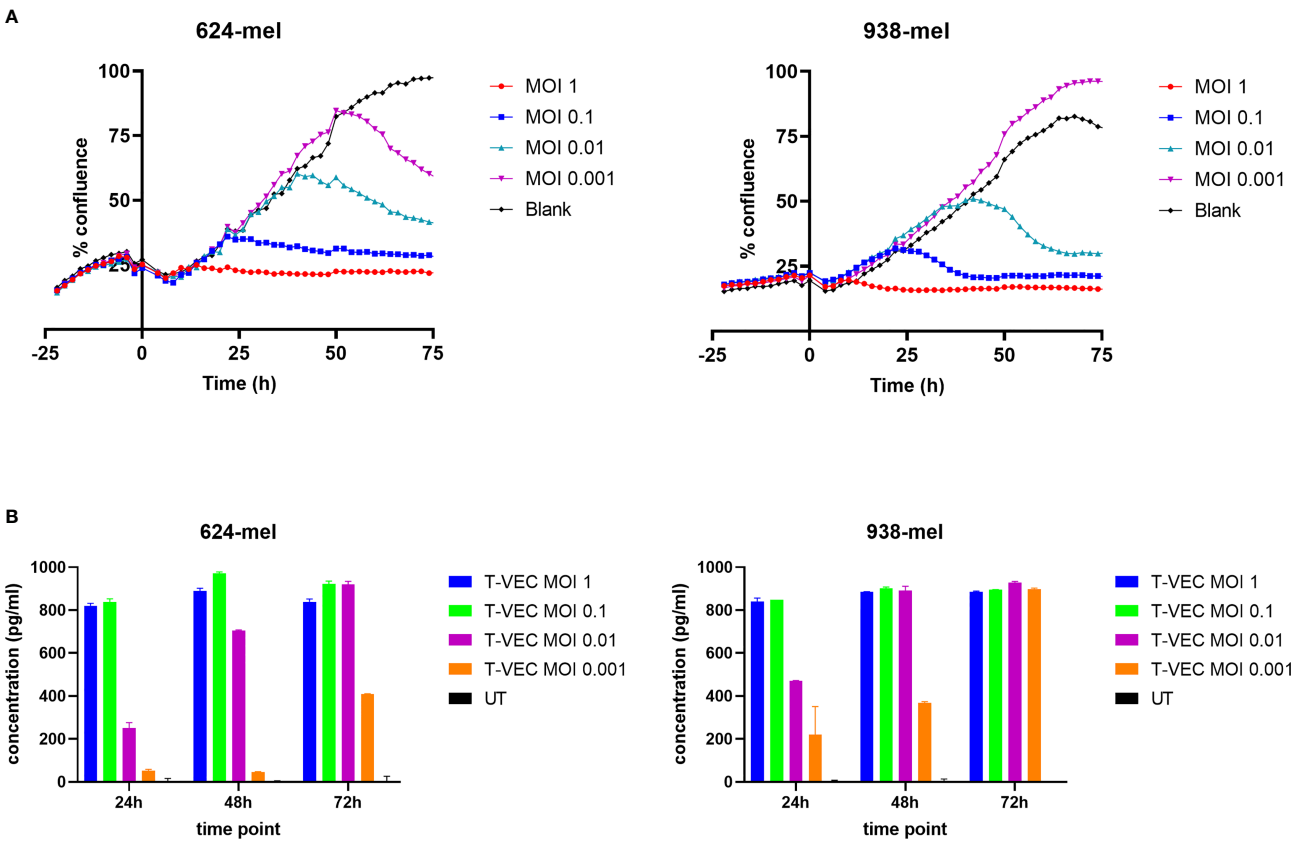

FIGURE 1 | T-VEC infection of melanoma cell lines. **(A)** The confluence of cells was analyzed in real time using the confluence image mask. T-VEC was added at the indicated MOIs at time 0. The left panel shows data for the 624-mel cell line and the right panel for the 938-mel cell line. Data points depict the mean of 3 replicate wells and the graphs show 1 representative experiment out of 3. **(B)** The secretion of GM-CSF after treatment with T-VEC at the indicated time points was measured by ELISA. The left panel shows data for the 624-mel cell line and the right panel for the 938-mel cell line. Data points depict the mean ± SD of 2 replicate wells and the graphs show 1 representative experiment out of 3. UT, untreated. Statistics left panel (624-mel): At 24h, all comparisons between the different conditions were highly significant ($p < 0.0001$), except for UT vs T-VEC MOI 0.001 ($p = 0.0123$) and T-VEC MOI 1 vs T-VEC MOI 0.1 ($p = 0.6944$, ns). At 48h, all comparisons between the different conditions were highly significant ($p < 0.0001$), except for UT vs T-VEC MOI 0.001 ($p = 0.0302$) and T-VEC MOI 1 vs T-VEC MOI 0.1 ($p = 0.0002$). At 72h, all comparisons between the different conditions were highly significant ($p < 0.0001$), except for T-VEC MOI 1 vs T-VEC MOI 0.1 ($p = 0.0002$), T-VEC MOI 1 vs T-VEC MOI 0.01 ($p = 0.0002$) and T-VEC MOI 0.1 vs T-VEC MOI 0.01 ($p > 0.9999$, ns). Statistics right panel (938-mel): At 24h, all comparisons between the different conditions were highly significant ($p < 0.0001$), except for UT vs T-VEC MOI 0.001 ($p = 0.0001$) and T-VEC MOI 1 vs T-VEC MOI 0.1 ($p = 0.9988$, ns). At 48h, all comparisons between the different conditions were highly significant ($p < 0.0001$), except T-VEC MOI 1 vs T-VEC MOI 0.1 ($p = 0.9913$, ns), T-VEC MOI 1 vs T-VEC MOI 0.01 ($p = 0.9998$, ns) and T-VEC MOI 0.1 vs T-VEC MOI 0.01 ($p = 0.9987$, ns). At 72h, all comparisons with the UT condition were highly significant ($p < 0.0001$), but differences between all other conditions were non-significant.

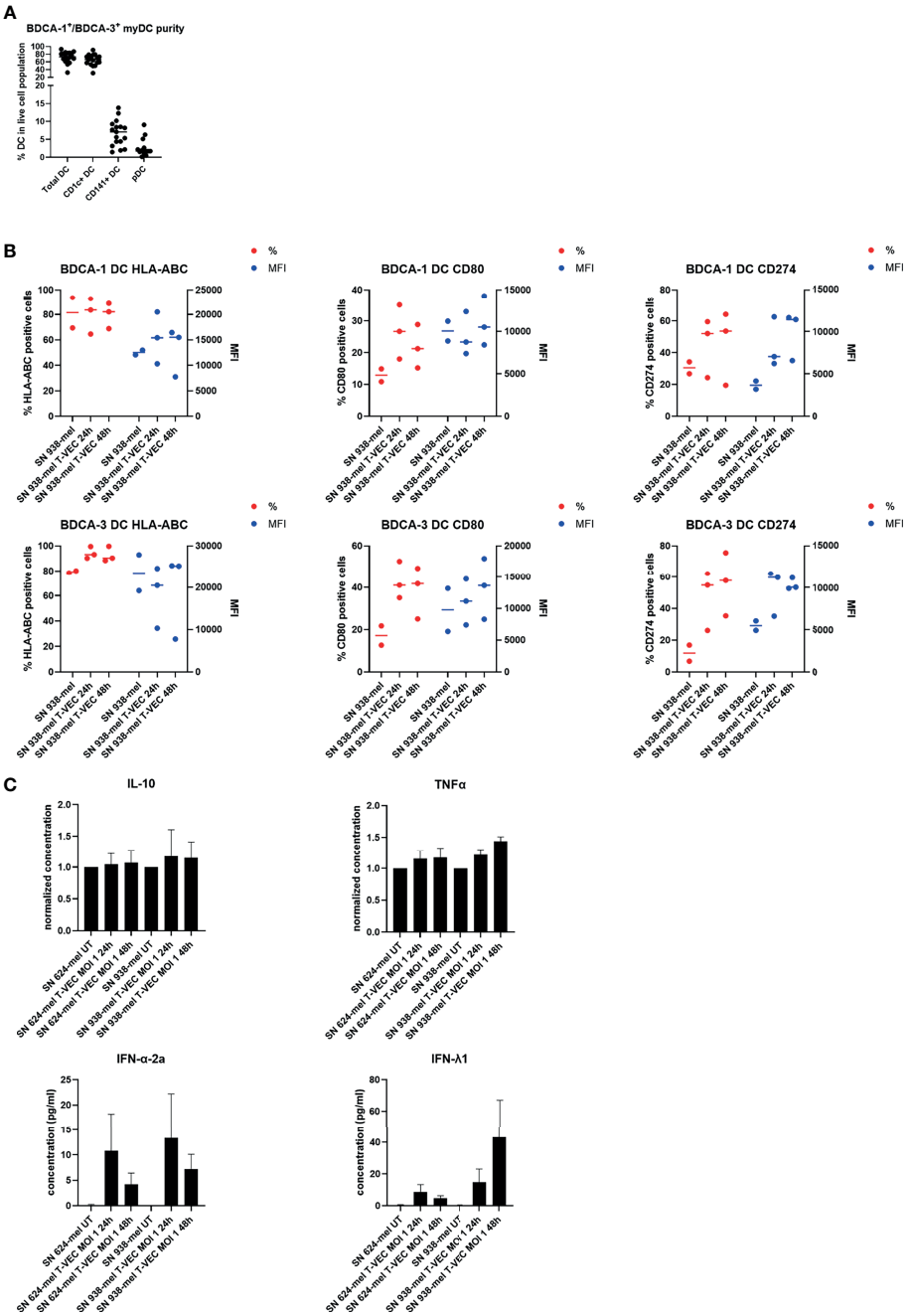

FIGURE 2 | Effect of supernatant from T-VEC-treated melanoma cells on purified BDCA-1$^+$/BDCA-3$^+$ myDC. **(A)** The purity of the purified BDCA-1$^+$/BDCA-3$^+$ myDC was analyzed by flow cytometry. This graph depicts the percentage of each DC subpopulation in the live cell population. **(B)** BDCA-1$^+$/BDCA-3$^+$ myDC were incubated for 24h with supernatant from untreated 938-mel cells, supernatant from 938-mel cells treated with T-VEC (MOI 1) during 24h or supernatant from 938-mel cells treated with T-VEC (MOI 1) during 48 h. After 24 h, the phenotype of the DC was analyzed by flow cytometry. On the left y-axis (red symbols) the percentage of expression of each marker is depicted and on the right y-axis (blue symbols) the mean fluorescence intensity (MFI) is plotted. Each symbol represents the result of an independent experiment, and the line represents the median. **(C)** On the supernatants from the cultures described in **(B)**, cytokine content was analyzed using MesoScale Diagnostics assays. For IL-10 and TNFα, concentrations were normalized to the conditions with supernatant from untreated tumor cells (either 624-mel or 938-mel) because the absolute cytokine concentrations showed high variation among donors. For IFN-α-2a and IFN-λ1, the actual concentrations are plotted. The graphs depict mean values ± standard error of mean (SEM) and represent data from 6 independent experiments.

Data for all markers and using another cell line 624-mel are shown in the **Supplementary Figure 1**. The supernatant from these myDC cultures was subsequently analyzed for its cytokine content and we observed a trend towards increased TNF-α, IFN-α-2a and IFN-λ1 concentrations upon culture in the presence of supernatant from T-VEC-treated melanoma cells, while IL-10 concentration remained unchanged (**Figure 2C**) and IL-12p70 was mainly undetectable (data not shown). These data show that the supernatant of T-VEC-treated melanoma cells contains inflammatory mediators that can induce a partial maturation in BDCA-1$^+$/BDCA-3$^+$ myDC. Since T-VEC might still be present in the supernatant from tumor cells, we also heat-inactivated the supernatant before addition to the BDCA-1$^+$/BDCA-3$^+$ myDC culture. We indeed observed a decreased cytokine production upon heat inactivation, but this effect was also observed in conditions without T-VEC (**Supplementary Figure 2**). To explore the immunostimulatory properties of T-VEC itself, we also analyzed the direct effect of T-VEC on BDCA-1$^+$/BDCA-3$^+$ myDC. T-VEC treatment did not exert an effect on the viability of BDCA-1$^+$/BDCA-3$^+$ myDC (data not shown), indicating it is not toxic. On BDCA-1$^+$ DC, we observed trends towards a decrease in CD83 and an increase of CD274 expression upon treatment with T-VEC. On BDCA-3$^+$ DC, we noted a trend to increased levels of HLA-ABC, CD83 and CD274 expression (**Figure 3A**). All other markers remained unchanged (**Supplementary Figure 3**). We also analyzed the cytokine secretion by BDCA-1$^+$/BDCA-3$^+$ myDC upon T-VEC treatment and noted an upregulation of IL-12p70, IFN-α-2a and IFN-λ1 (**Figure 3B**), although not statistically significant. These effects of T-VEC were abolished by heat inactivation before addition to the myDC cultures. These data show that the observed partial maturation effect of supernatant from T-VEC-treated melanoma cells is not completely mirrored by the effect of T-VEC itself. Moreover, since both T-VEC and supernatant from T-VEC-treated melanoma cells only show a trend towards a partial maturation of BDCA-1$^+$/BDCA-3$^+$ myDC, when compared to the complete maturation induced by the positive control (R848 + poly(I:C)) (data not shown), these data suggest that the potency of BDCA-1$^+$/BDCA-3$^+$ myDC treated with supernatant from T-VEC-treated melanoma cells could be enhanced further by the addition of a maturation stimulus.

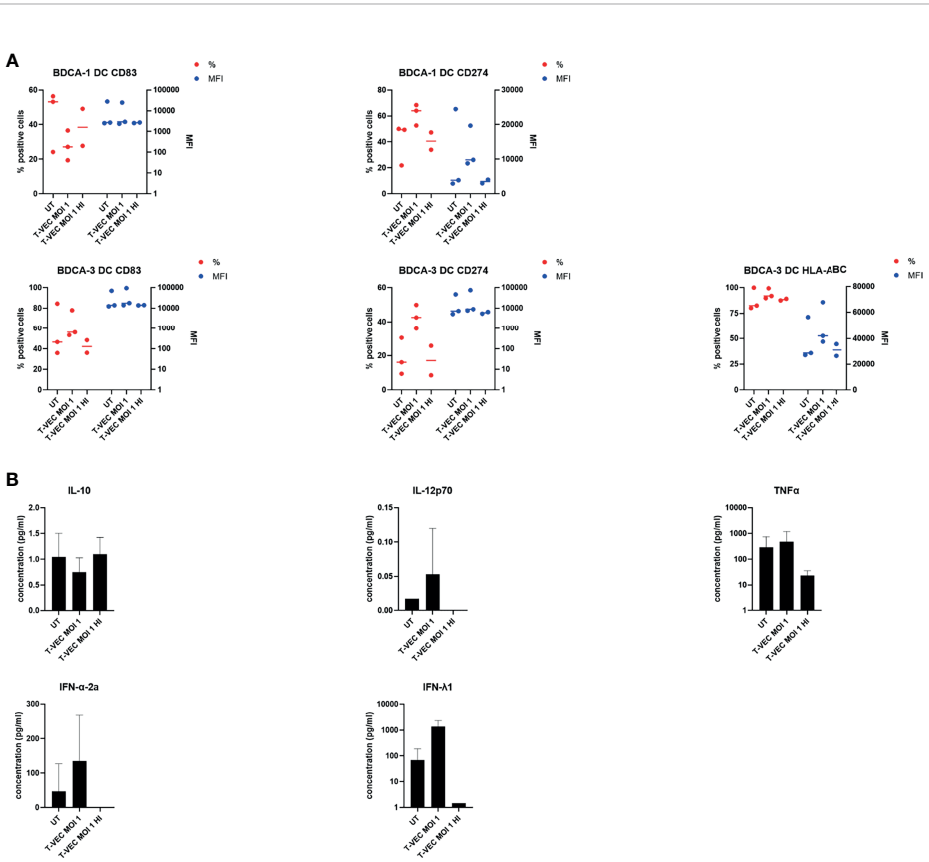

FIGURE 3 | Effect of T-VEC on purified BDCA-1$^+$/BDCA-3$^+$ myDC. **(A)** BDCA-1$^+$/BDCA-3$^+$ myDC were incubated with T-VEC (MOI 1), heat-inactivated T-VEC (MOI 1) or left untreated. After 24h, the phenotype of the DC was analyzed by flow cytometry. On the left y-axis (red symbols) the percentage of expression of each marker is depicted and on the right y-axis (blue symbols) the mean fluorescence intensity (MFI) is plotted. Each symbol represents the result of an independent experiment and the line represents the median. **(B)** On the supernatants from the cultures described in **(B)**, cytokine content was analyzed using MesoScale Diagnostics assays. The graphs depict mean values ± SD and represent data from 3 independent experiments. For IL-12p70, some values were undetectable.

T-VEC-Treated Melanoma Cells Are Efficiently Taken up by BDCA-1$^+$/BDCA-3$^+$ myDC

In order to investigate whether dying tumor cells, due to T-VEC treatment are taken up by BDCA-1$^+$/BDCA-3$^+$ myDC, we labeled the tumor cells with a pH-sensitive dye (pHrodo^{TM} dye) before co-culturing the cells with BDCA-1$^+$/BDCA-3$^+$ myDC. The fluorochrome only becomes fluorescent in an acidic environment, so when the labeled cells are engulfed by DC and enter the acidic phagosome, the fluorescent signal becomes detectable. We co-cultured pHrodo^{TM}-labeled, T-VEC-treated 938-mel cells with BDCA-1$^+$/BDCA-3$^+$ myDC at different ratios (tumor cell:DC of 0:1, 1:1, 2:1 and 5:1) and during different incubation times (2-4-6h). Uptake of dying cells was assessed by flow cytometry. As shown in **Figure 4**, for each DC population (CD11c$^+$ DC, CD1c$^+$ DC and CD141$^+$ DC), we noted an increased uptake over time as well as a higher uptake upon higher T:DC ratios. There was a trend towards a higher uptake by CD141$^+$ DC compared to CD1c$^+$ DC or CD11c$^+$ total DC population, but this was not statistically significant. These data show that BDCA-1$^+$/BDCA-3$^+$ myDC are capable of engulfing cellular material from dying tumor cells.

BDCA-1$^+$/BDCA-3$^+$ myDC Co-Cultured With Dying T-VEC-Treated Melanoma Cells Cross-Present Tumor Antigens to T Cells

Next, we explored whether BDCA-1$^+$/BDCA-3$^+$ myDC that are co-cultured with T-VEC-treated dying melanoma cells are capable of processing tumor antigens from the melanoma cells for presentation to T cells. To this end, we used BDCA-1$^+$/BDCA-3$^+$ myDC and T cells from an HLA-A2-positive patient. The T cells were transfected with mRNA encoding the α- and β-chain from the NY-ESO-1 TCR or the gp100 TCR to serve as a source of NY-ESO-1-specific and gp100-specific T cells respectively. HLA-A2-positive BDCA-1$^+$/BDCA-3$^+$ myDC were co-cultured during 24h with T-VEC-treated 938-mel cells (NY-ESO-1$^+$, gp100$^+$, HLA-A2$^-$) and subsequently, these DC were put in co-culture with the autologous NY-ESO-1-specific or gp100-specific T cells and after overnight incubation the production of IFN-γ was measured. **Figure 5** shows that BDCA-1$^+$/BDCA-3$^+$ myDC co-cultured with T-VEC-treated 938-mel cells indeed induce IFN-γ secretion from both the NY-ESO-1 TCR transfected T cells as well as from the gp100 TCR transfected T cells. The effect was highest using a DC:tumor cell ratio of 1:5, although it remained lower than DC pulsed with 10 nM peptide. Thus, BDCA-1$^+$/BDCA-3$^+$ myDC that have taken up T-VEC-treated dying tumor cells are capable of cross-presenting tumor antigens to tumor-specific T cells.

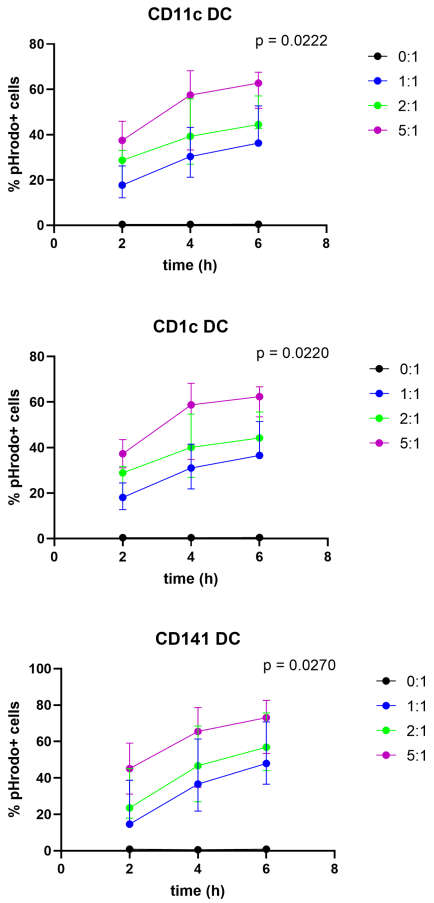

FIGURE 4 | Uptake of T-VEC-treated dying melanoma cells by BDCA-1$^+$/BDCA-3$^+$ myDC. 938-mel cells were treated with T-VEC at a MOI of 1 for 24h. Then, cells were harvested, counted and labeled with the pHrodo^{TM} Deep Red Mammalian and Bacterial Cell Labeling Kit. After labeling, labeled T-VEC-treated dying 938-mel cells were put in co-culture with purified BDCA-1$^+$/BDCA-3$^+$ myDC at the indicated ratios for 2, 4 or 6h. After co-culture for the indicated time points, cells were harvested and analyzed by flow cytometry. The different DC populations were gated and the percentage of pHrodo^{TM}-positive cells was determined. Each panel shows the uptake by the indicated DC population. Data are represented as mean ± SD from 3 independent experiments.

DISCUSSION

In this study, we investigated the interaction between T-VEC, an oncolytic HSV-1 virus, melanoma cells and naturally circulating myDC (BDCA-1$^+$ and BDCA-3$^+$). The mechanism of action of T-VEC exists of three phases (1): inducing a lytic cancer cell death with release of tumor-associated antigens and immune stimulatory molecules ("danger" signals) (2), attracting and activating immature DC through production of GM-CSF and (3) inducing an adaptive immune response against the cancer cells (19). We showed that the supernatant of T-VEC-treated melanoma cells can partially activate BDCA-1$^+$/BDCA-3$^+$ myDC and that these BDCA-1$^+$/BDCA-3$^+$ myDC can take up co-cultured T-VEC-treated melanoma cells. Finally, we were able to show that BDCA-1$^+$/BDCA-3$^+$ myDC that have taken up dying T-VEC-treated melanoma cells are able to cross-present tumor antigens to antigen-specific T cells. To our knowledge, these findings have not yet been described using BDCA-1$^+$/BDCA-3$^+$ myDC.

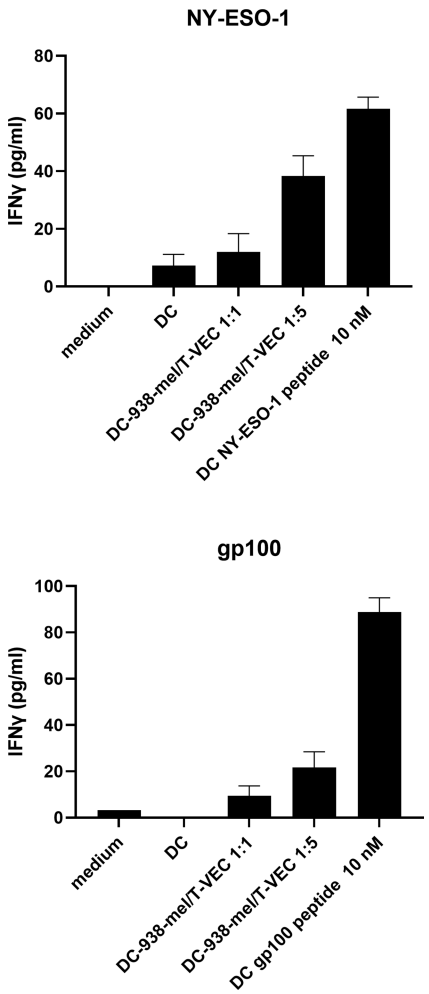

FIGURE 5 | Antigen presentation by BDCA-1$^+$/BDCA-3$^+$ myDC that have taken up T-VEC-treated melanoma cells. 938-mel cells were treated with T-VEC at a MOI of 1 for 24h and subsequently co-cultured with BDCA-1$^+$/BDCA-3$^+$ myDC from an HLA-A2$^+$ donor for 24h. After 24h, cells were harvested and counted and put into co-culture (1:1) with autologous T cells that were electroporated with mRNA encoding for the α- and β-chain of the TCR for NY-ESO-1 (top panel) or gp100 (bottom panel). After overnight co-culture of DC and TCR-transfected T cells, the supernatant was harvested and analyzed for IFN-γ content by ELISA. Data points depict the mean ± SD of 3 replicate wells.

Considering the effect of T-VEC on melanoma cell lines, our data confirms the findings that were reported previously by others: T-VEC effectively kills melanoma cells and a higher MOI results in a more rapid decrease of cell growth (17, 20). Other oncolytic viruses, such as Newcastle Disease Virus (NDV) have also been shown to be able to induce immunogenic apoptosis in the HER-2 positive human breast cancer cell line SK-BR-3 (21). However, we are the first to show the kinetics associated with T-VEC induced cell death. This allows future clinical trials to better estimate the optimal timing for administration of co-injectables such as DC or adjuvants. Except for the MOI 1 condition which induces almost immediate cell death, it takes 25-48 hours for T-VEC to induce cell death in melanoma cells.

Subsequently, we investigated the effect of both T-VEC itself as well as the supernatant from melanoma cell lines treated with T-VEC on myDC. T-VEC itself was not toxic for BDCA-1$^+$/BDCA-3$^+$ myDC. To investigate whether treatment of melanoma cells with T-VEC could result in the release of potent stimuli that could activate the injected DC, we *in vitro* treated BDCA-1$^+$/BDCA-3$^+$ myDC with supernatant from melanoma cells treated with T-VEC. We first focused on 'phenotypic maturation' by looking at the expression of classical maturation markers necessary for the activation of T cells such as CD40, CD80, CD86 and upregulation of HLA-ABC molecules. Next to this, we studied the release of cytokines that play a role in the anti-tumor immune response and have been shown to be produced by myDC. IL-10 is an immunosuppressive cytokine produced by a tolerogenic DC subset (22). IL-12 (with its functionally active heterodimer IL-12p70) promotes T cell differentiation into a Th1 effector type (T helper or cytotoxic T cell) (23). TNF-α is a pleiotropic cytokine with powerful immune stimulating functions. IFN-α-2a (type I interferon) and IFN-λ1 (type III interferon) are both induced in response to viral infections but also play a role in anti-tumor immunity. IFN-α-2a induces apoptosis in tumor cells and promotes NK and T-cell priming (24). IFN-λ has been discovered more recently and plays a role in triggering anti-tumor NK and T cells (25–29). Recently, it was shown that IFN-λ can be produced by BDCA-3$^+$ DC (30). It has been shown previously by Bai et al. that NDV infected MCF-7 cells have beneficial effects on the antigen presentation capacity of breast cancer patient derived dendritic cells demonstrated by an upregulation of CD40, CD80, CD83, CD86 and MHC class II (HLA-DR). Within these co-cultures, increased levels of IFN-α, IL-12 and IL-15 could be detected (31, 32). More recently, it was shown that NDV infected SK-BR-3 cells are able to mature monocyte derived dendritic cells as demonstrated by the upregulation of CD40, CD80, MHC class I and II, increased levels of several cytokines (IFN-α, IL-6, TNF-α, IL-12) and chemokines (MIP-1α, RANTES, IP-10) (21). Within this context, we observed a trend to increased TNF-α, IFN-α-2a and IFN-λ1 secretion as well as an increased expression of CD80, PD-L1 and HLA-ABC by the BDCA-1$^+$/BDCA-3$^+$ myDC after treatment with supernatant from T-VEC treated melanoma cells. Heat inactivation of the tumor cell supernatant before addition to myDC resulted in decreased cytokine production, but also in conditions without T-VEC. To rule out that part of this effect might be related to the presence of T-VEC itself, we also tested the effect of T-VEC alone, which also resulted in a slight increase of IFN-α-2a and IFN-λ1 production. However, in response to T-VEC alone we also detected the production of IL-12p70 which was not seen when using supernatant. Since we only noted a partial maturation of BDCA-1$^+$/BDCA-3$^+$ myDC, our data suggest that the additional co-injection of an adjuvant capable of maturing these myDC might even improve the functionality of the myDC. In our hands, heat-inactivated T-VEC was unable to induce this partial

DC maturation. This might indicate that either T-VEC needs to infect/enter DC to induce its maturing effect or alternatively, it could mean that important viral PAMPs are denatured by heat inactivation. The activation of PRR combined with cytokine signaling is needed to initiate a highly conserved cascade of genes regulated by transcription factors within DC, critical to control viral infections (33). It is currently not completely elucidated which PRR are involved in sensing of T-VEC, but for HSV-1, the virus from which T-VEC has been derived, it has been shown that multiple viral components can be recognized by different PRR (34). Viral proteins are recognized by TLR2 and herpes virus entry mediator (HVEM). Upon entry into the cell, viral DNA is sensed by TLR9 in endosomes, cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) in the cytoplasm, IFNγ inducible protein 16 (IFI16) mostly in the nucleus, and DNA dependent activator of IFN-regulatory factors (DAI). During replication, double stranded RNA is sensed by TLR3 in endosomes, melanoma differentiation-associated protein 5 (MDA5), RIG-I, and protein kinase RNA-activated (PKR) in the cytoplasm. Both HSV-1 and T-VEC have been shown to activate STING *via* the cGAS sensor (17, 35). It remains to be established which other PRR are involved in recognition of T-VEC.

It has already been shown that phagocytes such as macrophages or DC are able to take up material from dying tumor cells (21, 31, 32, 36–39). However, we are not aware that this has been shown before using natural BDCA-1^+/BDCA-3^+ myDC. We were able to show that each myDC subtype (both the complete $CD11c^+$ population, as well as the BDCA-1^+ and BDCA-3^+ subsets separately) was capable to engulf melanoma cells with a trend towards higher uptake by BDCA-3^+ DC which is expected as these cells are specialized in cross-presentation of (tumor) antigens (40).

It has been shown previously that monocyte-derived DC loaded with tumor antigens, derived from whole tumor cell lysates, are efficacious antigen-presenting cells able to initiate a T cell response against malignant glioma tumor cells showed by the upregulation of CD25 on $CD8^+$ T cells and by the generation of cytotoxicity against the target cells (39). Moreover, it has also been suggested that DC loaded with NDV-derived viral oncolysates might stimulate more potent T cell responses compared to DC pulsed with tumor lysate without NDV (31, 41). Hence, since myDC are known for their unique cross-presentation capabilities (4, 5), we were interested to see if the activated and loaded DC were subsequently able to cross-present melanoma-specific tumor antigens to antigen-specific T cells. To answer this question, we electroporated T cells with the T cell receptor for NY-ESO-1 and gp100, two melanoma-associated tumor antigens. We then co-cultured BDCA-1^+/BDCA-3^+ myDC with T-VEC-treated melanoma cells for 24 hours, followed by co-culture with the electroporated T cells. As we have demonstrated, BDCA-1^+/BDCA-3^+ myDC co-cultured with T-VEC-treated melanoma cells indeed induced secretion of IFN-γ by T cells, indicating that the melanoma antigens were indeed cross-presented, although at low levels compared to peptide-pulsed myDC. To our knowledge, we are the first to show that BDCA-1^+/BDCA-3^+ myDC are capable of cross-presenting tumor-associated antigens released by T-VEC treated melanoma cells to antigen-specific T cells.

At present, a phase I clinical trial is being conducted where T-VEC and myDC are co-injected intratumorally (ClinicalTrials.gov Identifier: NCT03747744). Early results from this trial indicate that durable tumor responses can be obtained in patients with immune checkpoint inhibitor refractory melanoma (42).

For future translation of this type of clinical trials involving academic cell therapy production to a larger patient population, several practical and logistic hurdles remain. There is an important need for exchange of best practices regarding transportation (to/from a GMP facility), cryopreservation, manipulation and manufacturing of cell therapy products. However, as the recent development and FDA approval of CAR-T cell therapies such as Yescarta® and Kymriah® have demonstrated it is not impossible to scale cell-therapy development and make it accessible to more patients on a global scale (43).

In the future, more research is needed regarding the combination of intratumoral treatment modalities such as T-VEC and (intratumorally injected) myDC. It is yet unclear how important the role of the induced cell lysis by T-VEC is *in vivo*. Next to this, we know that more potent maturation factors exist for these DC subtypes, but these remain as yet unavailable for clinical (intratumoral) use. Both clinical and pre-clinical validation of promising combinations is warranted for future combination therapies involving myDC.

In conclusion, we have shown that the combination of T-VEC with BDCA-1^+/BDCA-3^+ myDC is complementary and able to *ex vivo* induce an immune response against melanoma cells. We have shown that melanoma cells are killed after infection with T-VEC, resulting in the release of tumor antigens and partial maturation of BDCA-1^+/BDCA-3^+ myDC. These myDC are able to take up melanoma antigens, process these antigens and subsequently present these antigens to antigen-specific T cells. These experiments show that the combination of intratumoral injection of myDC and T-VEC are complementary treatment modalities that could lead to an adaptive immune response against melanoma and should be further explored in the clinical setting.

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 Ethics committee of the UZ Brussel. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BN, KB, and JA conceptualized the design of the study. JT, JDM, ID, JKS, LF, KB and ST were responsible for the experimental design. JT, IVR and ST acquired the data. JT and ST analyzed the data and drafted the manuscript. All authors critically revised the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.733506/full#supplementary-material

Supplementary Figure 1 | Effect of supernatant from T-VEC-treated melanoma cells on BDCA-1+/BDCA-3+ myDC. BDCA-1+/BDCA-3+ myDC were incubated for 24h with supernatant from untreated 624-mel cells, supernatant from 624-mel cells treated with T-VEC (MOI 1) during 24h, supernatant from 624-mel cells treated with supernatant from 624-mel cells treated with T-VEC (MOI 1) during 48h, supernatant from untreated 938-mel cells, supernatant from 938-mel cells treated with T-VEC (MOI 1) during 24h or supernatant from 938-mel cells treated with T-VEC (MOI 1) during 48h. After 24h, the phenotype of the DC was analyzed by flow cytometry. **(A)** Results gated on BDCA-1+ DC. **(B)** Results gated on BDCA-3+ DC. On the left y-axis (red symbols) the percentage of expression of each marker is depicted and on the right y-axis (blue symbols) the mean fluorescence intensity (MFI) is plotted. Each symbol represents the result of an independent experiment, and the line represents the median.

Supplementary Figure 2 | Effect of heat inactivation of supernatant from T-VEC-treated melanoma cells on BDCA-1+/BDCA-3+ myDC cytokine secretion. BDCA-1+/BDCA-3+ myDC were incubated for 24h with supernatant from untreated 938-mel cells, supernatant from 938-mel cells treated with T-VEC (MOI 1) during 24h or supernatant from 938-mel cells treated with T-VEC (MOI 1) during 48h. The supernatant was either added as such or after a heat-inactivation step. After 24h, the supernatant from the cultures was harvested and cytokine content was analyzed using MesoScale Diagnostics assays. The cytokine concentrations were normalized to the condition where the cells were incubated with the supernatant of untreated tumor cells (both for 624-mel and 938-mel). Each symbol represents the result of an independent experiment, and the bars indicate the mean ± SD.

Supplementary Figure 3 | Effect of T-VEC on BDCA-1+/BDCA-3+ myDC. BDCA-1+/BDCA-3+ myDC were incubated with T-VEC (MOI 1), heat-inactivated T-VEC (MOI 1) or left untreated. After 24h the phenotype of the DC was analyzed by flow cytometry. **(A)** Results gated on BDCA-1+ DC. **(B)** Results gated on BDCA-3+ DC. On the left y-axis (red symbols) the percentage of expression of each marker is depicted and on the right y-axis (blue symbols) the mean fluorescence intensity (MFI) is plotted. Each symbol represents the result of an independent experiment, and the line represents the median.

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Dendritic Cell Vaccination of Glioblastoma: Road to Success or Dead End

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Glioblastomas (GBM) are the most frequent and aggressive malignant primary brain tumor and remains a therapeutic challenge: even after multimodal therapy, median survival of patients is only 15 months. Dendritic cell vaccination (DCV) is an active immunotherapy that aims at inducing an antitumoral immune response. Numerous DCV trials have been performed, vaccinating hundreds of GBM patients and confirming feasibility and safety. Many of these studies reported induction of an antitumoral immune response and indicated improved survival after DCV. However, two controlled randomized trials failed to detect a survival benefit. This raises the question of whether the promising concept of DCV may not hold true or whether we are not yet realizing the full potential of this therapeutic approach. Here, we discuss the results of recent vaccination trials, relevant parameters of the vaccines themselves and of their application, and possible synergies between DCV and other therapeutic approaches targeting the immunosuppressive microenvironment of GBM.

Keywords: dendritic cells, vaccination, immunotherapy, glioblastoma, review (article), glioma, brain tumor

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INTRODUCTION

Glioblastomas (GBM) are highly invasive, malignant tumors of the central nervous system. According to the 2021 World Health Organization classification, GBM are grade 4 tumors, that belong to the group of adult diffuse gliomas (1). They lack mutations in the isocitrate-dehydrogenase (IDH) gene, which now discriminates GBM from IDH-mutated grade 4 astrocytomas, which have been regarded as secondary GBM before. Based on gene expression signatures, GBM can be further subdivided into mesenchymal, proneural, neural and classical subtypes (2).

Although representing the most frequent malignant primary brain tumor (~30%–40%), GBM are rare; the yearly incidence is three to four per 100,000 adults (3). Nevertheless, they are a highly fatal tumor, responsible for 2% of cancer-related deaths, with a yearly death rate of four to five per 100,000 adults. The established therapeutic standard of care in the first-line therapy for GBM combines maximal safe resection, fractionated radiotherapy with concomitant alkylating temozolomide (TMZ) chemotherapy, followed by adjuvant TMZ treatment. This multimodal approach has improved survival of patients significantly. Nevertheless, prognosis of newly diagnosed GBM patients is dismal. Median overall survival (mOS) is only 14.6 months, and the

2-year survival rate is 27.2% (4, 5). In GBM patients with unmethylated O^6-methylguanine-DNA-methyltransferase (MGMT) promoter, producing the DNA-repair enzyme, prognosis is even worse [methylated *vs.* unmethylated mOS is 21.7 *vs.* 12.7 months; (6)]. Disease recurrence is universal, there is no effective therapy for recurrent disease, and median survival after relapse is 6.2 months. Therapeutic alternatives include lomustine, carmustine, tumor-treating field (TTF) therapy, and the angiogenesis inhibitor bevacizumab (7–10). Thus, there is a clear need for novel therapeutic modalities in GBM.

Dendritic cells (DC) are professional antigen-presenting cells, which are key to the development of T-cell responses (11, 12). As immature (resting) cells, they reside in most tissues, where they sample antigens. When activated by pathological changes in the tissue, they migrate to the draining lymph nodes and present there as mature (activated) DC peptides processed from the antigenic material taken up in the tissue on human leukocyte antigen (HLA) class I and II molecules, in an immunostimulatory context of co-stimulatory and accessory molecules. Antigen-specific cytotoxic T lymphocyte (CTL) and helper T cells (T_H) recognizing these peptides get activated, proliferate, and differentiate to effector cells, which execute the various actions of cellular adaptive immune responses, including the killing of target cells. DC vaccination (DCV) is an active immunotherapy seeking to exploit this pivotal role of DC therapeutically: patients are vaccinated with tumor-associated antigens (TAA)-loaded DC, with the concept that they migrate to local lymph nodes, present TAA-derived peptides on HLA molecules, and initiate an antitumoral T-cell response, which selectively kills the tumor cells and prevents tumor recurrence, due to immunological memory (13, 14).

DCV was first evaluated in 1996 in a clinical trial for B-cell lymphoma (15). In 1999, Dhodapkar et al. reported the induction of antigenic target-directed T-cell responses by DCV (16). The authors vaccinated nine healthy individuals with mature DC loaded with an influenza matrix peptide, keyhole limpet hemocyanin, or tetanus toxoid and showed induction of target-specific T-cell immunity after a single application of the vaccine. The clinical efficacy of DCV was documented in 2006 in a phase III trial in patients with hormone refractory prostate cancer (17). Patients were treated with either placebo (leukocytes) or DC loaded with a fusion protein of prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Provenge/sipuleucel-T). mOS of vaccinated patients was significantly improved compared with that in the placebo-treated control group (25.9 *vs.* 21.4 months), results that were confirmed in a second trial (18); and Provenge/sipuleucel-T was the first (and so far only) DC vaccine approved by the U.S. Food and Drug Administration in 2010 (19). In GBM, hundreds of patients have been vaccinated, mainly in smaller uncontrolled trials. Although the results are promising, few studies can provide robust evidence, and overall the efficacy of DCV in GBM is variable, ranging from little or no clinical response to significant response. Therefore, we address the questions of which parameters could possibly effect efficacy of DCV and whether and how it may be possible to improve it.

DENDRITIC CELL VACCINATION FOR GLIOBLASTOMA IN ANIMAL MODELS

Already in 1999, Liau and colleagues documented in the 9L rat glioma model that vaccination with DC pulsed with acid-eluted peptides derived from 9L glioma cells can prolong survival of glioma-bearing animals (20). In addition, vaccination was associated with infiltration of tumors with $CD8^+$ and, to a lesser extent, $CD4^+$ T cells and the development of glioma 9L cell-specific CTL responses. In 2000, Heimberger et al., who had vaccinated mice with DC pulsed with lysates derived from the spontaneously arising 560 glioma cell line, which had been transfected with the murine homolog of the mutated epidermal growth factor receptor variant III (EGFRvIII) (21), reported that vaccination could protect mice from subsequent intracranial tumor challenge. Survival of vaccinated animals was significantly prolonged compared with that in control animals receiving unpulsed DC. The surviving animals showed antitumoral memory and were healthy, were neurologically normal, and showed no signs of autoimmune encephalitis. Again, vaccination was associated with the development of glioma-cell-specific CTL responses, but interestingly, those were not directed against EGFRvIII, but against other unknown TAA.

Meanwhile, numerous animal studies have been performed in prophylactic (22–24) and curative DCV settings (25–31). Overall, there is clear evidence from these initial animal studies that DCV reduces tumor growth, can prolong survival, induces tumor-specific IFNγ and CTL responses, is associated with T-cell infiltration of tumors, particularly by $CD8^+$ T cells, and results in long-lasting antitumoral memory that provides protection from tumor re-challenge. At the same time, vaccination appears to be safe and not to be associated with the development of autoimmunity. Thus, animal studies provided a proof-of-principle for DCV of GBM. Moreover, they continue to contribute to the development of vaccination strategies to increase efficacy (32).

TREATMENT OF GLIOBLASTOMA PATIENTS WITH DENDRITIC CELL VACCINATION

Active immunotherapy with DCV has been pioneered by Liau et al., who described the vaccination of a GBM patient with recurrent disease with DC pulsed with eluted peptides of an HLA class I-matched GBM cell culture in 2000 (33). Although induction of an anti-peptide immune response was observed, the patient progressed and died 3 months later. Since then, numerous studies have been published (**Table 1**), including six controlled, randomized trials (60, 62, 76, 79, 81, 83), and several more are underway (88). Patients included mainly adults, but also children and adolescents (34, 38, 39, 41, 48, 52, 61, 64, 65, 69, 78), and the age of vaccinated patients varied between 1 and 80 years.

TABLE 1 | Concluded clinical trials and case reports on dendritic cell vaccination of glioblastoma patients.

Diagnosis All/ nd GBM/ rec GBM control	**Antigenic target**	**[7]DC maturation**	**DC application**	**DC dose** number vaccines/ cells/vaccine	**Clinical outcome (GBM)** OS/PFS/ others	**Immunological responses (GBM)** DTH IFNg[10] others[11]	**Toxicity[9]**	**Ref**
rec GBM 1/0/1	Eluted peptides	-	i.d.	3× biweekly 5×10^6	PD	- - 1	None	(33)
rec HGG 8/0/5	HGG/DC fusion	TNFα	i.d.	1–8× triweekly $2.4–8.8 \times 10^6$	1× MR, 1× SD	- 5/5 -	Erythema	(34)
nd HGG 9/7/0	Eluted peptides	-	s.c.	3× biweekly 1×10^6	nd: 15.0 m/–	- - 4/7[12]	Fever, lymph node swelling, vomiting/ nausea	(35)
nd/rec GBM 42/24/18	Tumor lysate	–	s.c.	3× biweekly ± 1× after 6 weeks $10–40 \times 10^6$				(36)
rec HGG 10/0/7	Tumor lysate	-	i.d. + i.t.	1–10× triweekly $10–32 \times 10^6$	rec[8]: 17.7 m/– 4× SD	3/6 2/4 -	Headache, erythema	(37)
rec HGG 9/0/2	Tumor mRNA	–	i.d. + i.v.	3× biweekly ± 3× monthly $5 \times 10^6/m^2$ (i.v.) + 5×10^6 (i.d.)	PD	- 0/4[12] 0/3[12]	None	(38)
rec GBM 1/0/1	Tumor lysate	TNFα, IL-1β, PGE2	i.d.	2× biweekly + 4× monthly $1–9 \times 10^6$	CR (2 years)		None	(39)
rec HGG 15/0/6	HGG/DC fusion	TNFα	i.d.	3× biweekly $3.6–32.3 \times 10^6$	rec[8]: 8.5 m/– 1× SD (4 m)	- 0/6 0/8	Fever, seizure, erythema, transient liver dysfunction, lymphopenia	(40)
rec HGG 12/0/7	Tumor lysate	TNFα, IL-1β, PGE2	i.d.	2× biweekly + 4× monthly $0.8–18 \times 10^6$	1× CR	6/8[12] - -	Peritumoral edema (grade 4), morning stiffness, hematotoxicity, nocturnal sweating, meningeal irritation	(41)
nd/rec GBM 25/11/14	Tumor lysate/ eluted peptides	–	s.c.	3× biweekly ± 1× after 6 weeks $10–40 \times 10^6$	nd: 34.4 m/– rec: 29.6 m/–	- 40%–60%[12] 60%[9]		(42)
nd/rec HGG 14/1/9	Tumor lysate	-	s.c.	3× biweekly $10–100 \times 10^6$	rec: 30.6 m/–	- nd: 0/1; rec: 4/5 nd: 0/1; rec: 2/6	Headache, fatigue, erythema, seizure	(43)
nd/rec GBM 12/6/6	Eluted peptides	–	i.d.	3× biweekly 1, 5 or 10×10^6	nd[8]: 27.9 m/16.3 m rec[8]: 16.6 m/12.5 m	- - nd: 4/6; rec: 2/6	Fever, flu-like, fatigue, myalgia, nausea/ vomiting, erythema, itching, lymph node swelling, diarrhea/constipation	(44)
rec HGG 24/0/18	Tumor lysate	- or OK432	i.d. + i.t.	1–22× (i.d.) 0-18× (i.t.) triweekly $1–32 \times 10^6$	rec[8]: 15.5 m/– 2× MR, 2× SD	8/15 6/13 -	Headache, erythema	(45)
rec GBM 1/0/1	Tumor lysate	–	i.v.	5× biweekly			Fever	(46)
nd GBM 6/6/0	Tumor lysate	TNFα, IL-1β, IFNγ	i.d.	2× biweekly 2×10^6	nd[8]: –/6.0 m	- 0/5 -	Headache	(47)
rec GBM 56/0/56	Tumor lysate	TNFα, IL-1β, PGE2	i.d.	3–7×: 2 biweekly + others monthly or 3–9× biweekly or 4× weekly $0.7–25.7 \times 10^6$	rec: 9.6 m/3.0 m	9/17 - -	Peritumoral edema (grade 4), hematotoxicity, hemiparesis, dysphasia, headache, vomiting, flu-like, seizure, fatigue, myalgia, hygroma, intratumoral hemorrhage, erythema	(48)
nd/rec HGG 13/7/2	Irradiated tumor cells	MCM	i.d.	2–13×: 6× biweekly + every 6 weeks 1×10^6	nd[8]: 11.0 m/– rec[8]: 5.0 m/–		None related to DCV	(49)
nd/rec HGG 44/11/23	Tumor lysate	–	s.c.	3× biweekly + 1× after 6 weeks $10–40 \times 10^6$		- 17/34[12] -	No grade 3/4	(50)

(Continued)

TABLE 1 | Continued

Diagnosis All/ nd GBM/ rec GBM control	**Antigenic target**	**[7]DC maturation**	**DC application**	**DC dose** number vaccines/ cells/vaccine	**Clinical outcome (GBM)** OS/PFS/ others	**Immunological responses (GBM)** DTH IFNg[10] others[11]	**Toxicity[9]**	**Ref**
nd GBM 12/12/0	EGFRIII peptide-KLH conjugate	TNFα, IL-1β, IL-6	i.d.	3× biweekly $30–100 \times 10^6$	nd: 22.8 m/10.2 m	5/9 - 10/12	Increased erythrocyte sedimentation rate, increased rheumatoid factor level	(51)
rec HGG 45/0/23	Tumor lysate	TNFα, IL-1β ± PGE2	i.d.	3–7×: 2 biweekly + others monthly or 4–16× biweekly or 4× weekly $0.5–23.8 \times 10^6$	rec[8]: 12.2 m/4.3 m		Fatigue, headache, fever, itching, vomiting, flu-like	(52)
nd GBM 8/8/0	Tumor lysate	TNFα, IL-1β, PGE2	i.d.	4× weekly $2–24 \times 10^6$	nd: 24.0 m/18.0 m	2/7 5/8 -	Lymphopenia, focal epileptic insult, dysphasia, fatigue, malaise, myalgia, ischemic event (grade 4), hematotoxicity (grade 3), status epilepticus (grade 4)	(53)
nd/rec HGG 17/8/6	Heat-shocked irradiated cells	-	s.c.	4× weekly + 2× biweekly + 4× monthly $10–60 \times 10^6$	nd[8]: 12.1 m/– rec[8]: 31.8 m/–		Lymphopenia (grade 3/4), transient hepatic dysfunction, seizure, hydrocephalus, anemia, myalgia, skull wound infection	(54)
nd GBM 10/10/0	Tumor lysate	TNFα, PGE2	i.n.	3× biweekly 30×10^6	nd: 28.0 m/9.5 m	0/10 4/10 -	Neck pain	(55)
rec HGG 22/0/13	Peptides[1]	TNFα, IL-1β, IFNα, IFNγ, poly(IC)	i.n.	4× biweekly + 5× monthly $10 \text{ or } 30 \times 10^6$	rec[8]: 12.0 m/4.0 m 1× CR (>13 m), 1× PR	- 5/12 5/10	Erythema, flu-like, fatigue, myalgia, fever, chill/rigor, headache, lymphopenia	(56)
nd/rec GBM 23/15/8	Tumor lysate	–	i.d.	3× biweekly ± ≤10× 3-monthly $1 \text{ or } 5 \times 10^6$	nd: 35.9 m/– rec: 17.9 m/–		Fatigue, nausea/vomiting, diarrhea, arthralgia, fever, lymphadenopathy, erythema, myalgia, shingles, allergic rhinitis, pruritus, headache, constipation, heartburn, dermatitis/rash, anorexia, abdominal pain	(57)
rec HGG 9/0/7	Peptides[2]	TNFα, IL-1β, IFNα, IFNγ, poly(IC)	i.d.	4× weekly ± ≤ 6× $10, 20, \text{or } 50 \times 10^6$	PD	4/7 4/6 -	Transient hepatic dysfunction	(58)
nd GBM 77/77/0	Tumor lysate	TNFα, IL-1β, PGE2	i.d.	4× weekly $0.24–55 \times 10^6$	nd: 18.3 m/10.4 m		Fatigue, rash/itching, shoulder pain, anorexia, myalgia, nausea/vomiting, seizure, confusion, humerus fracture, lethargy, ectopic cerebral lesion, depression, dysphasia, esophagitis, otitis media serosa, lymphopenia, leukopenia; grade 3/4 seizure, allergic reaction to TMZ, cerebral abscess, deep vein thrombosis, hydrocephalus, ischemic bowel perforation, lung and peripheral edema, osteoporotic fracture, dementia, focal status epilepticus, ischemic stroke, status epilepticus, thrombocytopenia, lymphopenia, leukopenia; grade 5: overwhelming infection	(59)
nd GBM 18/18/0 16	Tumor lysate	-	s.c.	4× weekly + 2× biweekly + 4× monthly $20\text{-}50 \times 10^6$	nd: 31.5 m/8.5 m		Hepatic dysfunction, lymphopenia, hemiplegia, pancytopenia, intracranial pressure, nausea/vomiting	(60)
rec HGG 8/0/5	IL-13Rα2 peptides	TNFα, IL-1β, IL-6	i.d.	2–6× biweekly 10×10^7	rec[8]: 7.0 m/– 1× MR, 2× SD (2-4 m)	- 2/3 2/3	Fatigue, erythema	(61)

(Continued)

TABLE 1 | Continued

Diagnosis All/ nd GBM/ rec GBM control	**Antigenic target**	**[7]DC maturation**	**DC application**	**DC dose** number vaccines/ cells/vaccine	**Clinical outcome (GBM)** OS/PFS/ others	**Immunological responses (GBM)** DTH IFNg[10] others[11]	**Toxicity[9]**	**Ref**
nd GBM 13/13/0 12	Tumor lysate	TNFα, IL-1β, PGE2	s.c.	2× weekly + 2× biweekly 6×10^6	nd: 17.0 m/11.9 m 3× CR (89 m), 6× PR, 1× SD (9 m)	- Increase -	Fever, erythema	(62)
nd GBM 5/5/0	Tumor lysate	TNFα, IFNα, poly(IC)	?	4–14: 2× bimonthly + 2× monthly + 4× bimonthly + others quarterly $0.8–10 \times 10^6$	nd: 27.4 m/16.1 m	- - 3/3	Seizure	(63)
nd HGG 7/5/0	Tumor lysate	-	i.d.	2–4 biweekly 1×10^6		- - -	Headache, injection site erythema, elevated alkaline phosphatase (grade 4)	(64)
rec GBM 15/0/15	Tumor lysate	TNFα IL-1β IL-6 PGE2	i.d.	3–4 × biweekly 2× monthly $1/2 \times 10^7$	rec: 8 m/4.4 m	- Increase NK cell response	Ependymitis/hydrocephalus, anemia grade 2, fever, cutaneous induration, cutaneous flushing, seizures, cerebral edema, tumor bleeding	(65)
nd/rec GBM 19/16/3	Peptides[3]	TNFα	i.d.	3× biweekly 10×10^6	nd: 38.4 m/16.9 m	- 5/15[12] 5/15[12]	Diarrhea, fatigue, flushing, pruritus, rash, vomiting	(66)
nd/rec HGG 27/23/4	Tumor lysate/ peptides	–/ TNFα, IL-1β, IL-6, PGE2	i.d.	3× biweekly/3× biweekly + 3× monthly	nd 34.4 m/18.1 m rec: 14.5 m/9.6 m	- - Increase	Grade 1–2 flu-like (headache, low-grade fever, nausea, vomiting, fatigue), injection site reactions, lymphadenopathy, rashes	(67)
nd GBM 7/7/0	GBM CSC mRNA	TNFα, IL-1β, IL-6, PGE2	i.d.	9–18×: 2×/week + 3× weekly + others monthly 10×10^6	nd: 25.0 m/22.8 m	1/7 - 7/7	Fatigue, anorexia, nausea, seizure, constipation, fatigue (grade 3)	(68)
rec HGG 8/0/6	Apoptotic bodies allogeneic CSC GBM6-AD	TNFα, IL-1β, IFNα, IFNγ, poly(IC)	s.c.	1–9×: 5× biweekly + 5× monthly	3× SD	- 0/6[13] 3/6[13]	Fatigue, erythema, induration (grade 1)	(69)
rec GBM 14/0/14	Irradiated tumor cells	MCM	i.d.	3× biweekly 6× monthly 4×10^6/ 1×10^6	rec[8]: 23.0 m/5.0 m 2× PR, 1× SD (31.5 m)	0/14 2/9 -	Nausea/vomiting, headache, seizure, thrombocytopenia, syncopal event (grade 3), bilateral cataracts (grade 3)	(70)
nd GBM 13/13/0	CMVpp65 mRNA	TNFα, IL-1β, IL-6, PGE2	i.d.	3× biweekly + others monthly 20×10^6	nd: 18.5 m/10.8 m	- Durable -	None related to vaccine	(71)
rec HGG 10/0/6	WT-1 peptides/ tumor lysate	OK432, PGE2	i.d.	5–7× biweekly + ≤23× $7–99.4 \times 10^6$	rec: 18.0 m/– 1× SD	5/5 - 3/4	Erythema, fever, fatigue	(72)
nd/rec GBM 32/22/10	GBM/DC fusion	TNFα	i.d.	3× monthly + 6/12-monthly $0.72–2.5 \times 10^6$	nd: 30.5 m/18.3 m rec: 18 m/10.3 m	- Positive 4/4	Injection site reaction	(73)
nd GBM 11/11/0	CMVpp65 mRNA	TNFα, IL-1β, IL-6, PGE2	i.d.	10× biweekly and monthly 20×10^6	41.1 m/25.3 m	- 10/11 Increase	No AE related to DCV except for GM-CSF autoantibody (grade 3)	(74)
nd GBM 32/32/0	Tumor lysate	TNFα, IFNα, poly (IC)	i.d.	>6 10×10^6	23.4 m/12.7 m	- 8/25 No change	No AE related to DCV	(75)
nd GBM 34/34/0 42	Tumor lysate	IFNγ, LPS	i.n.	up to 15×: 4× weekly, 5× monthly, 3-monthly $1–5 \times 10^6$	18.8 m/6.8 m		More AE in DCV group; more TMZ related AE in DCV group (thrombocytopenia more frequent in DCV); related to DCV: local pain, local reactions, fever, joint pain, general weakness	(76)

(Continued)

TABLE 1 | Continued

Diagnosis All/ nd GBM/ rec GBM control	Antigenic target	[7]DC maturation	DC application	DC dose number vaccines/ cells/vaccine	Clinical outcome (GBM) OS/PFS/ others	Immunological responses (GBM) DTH IFNg[10] others[11]	Toxicity[9]	Ref
nd GBM 43/43/0 42	Tumor lysate	IFNγ, LPS	i.n.	Up to 15×: 4× weekly, 5× monthly, 3-monthly 1–5 × 10^6		- Increase Increase		(77)
nd GBM 27/27/0 20	Tumor lysate of irradiated tumor cells	-		10×: 4× biweekly, 6× monthly 20–50 × 10^6	31 m/–			(78)
nd GBM 232/232/0 99	Tumor lysate	–	i.d.	3× every 10 days, 3× monthly, every 6 months 2.5 × 10^6	23.1 m/–		2.1% of pts grade 3/4 AE related cerebral edema, seizures, nausea, lymph gland infection; non-serious: injection site reactions, fatigue, low-grade fever, night chills	(79)
nd GBM 24/24/0	Tumor lysate	TNFα, IL-1β, IL-6, PGE2	i.d.	4× biweekly, 2× monthly, 1× 2 month later 5/10 × 10^6	20.1 m/10.5 m	- Increase NK cell response	1 pulmonary embolism, 1 deep venous thrombosis + embolism, 1 disseminated intravascular coagulation, seizures, convulsion, myositis, skin reaction with itching, erythema, urticaria, inflammation	(80)
nd/rec GBM 22/13/9 21	CSC lysate	–	i.d.	3× weekly 2–4 × 10^6	13.7 m/6.9 m[12]	- Increase -	Mild fever, erythema	(81)
rec GBM 20/0/20	Tumor lysate	TNFα, IL-1β, IL-6, PGE2	i.d.	3× biweekly, 2× monthly 20/10/5 × 10^6		- Increase -	Brain edema, vomiting, asthenia, seizure, dysphasia, dizziness, cognitive disturbance, hyposthenia, vaccination site reaction: erythema, pruritus, pain, induration	(82)
nd GBM 81/81/0 43	Peptides[4]	IFNγ, LPS	i.d.	4× weekly, 4× monthly, every 6 months 11 × 10^6	17 m/11.2 m	- 34/68 -	No AE related to DCV; fatigue, convulsions, nausea	(83)
nd GBM 23/23/0	CMVpp65 mRNA	TNFα, IL-1β, IL-6, PGE2	i.d.	3× biweekly, monthly 20 × 10^6	41.1/41.4 m/–			(84)
nd HGG 16/14/0	Peptides[5]	TNFα, IL-1β, IFNα, IFNγ, poly (IC)	i.d.	3× weekly, 2× biweekly, 5× monthly 10–50 × 10^6	19 m/11 m	27% 67% -	Only ≤grade 3	(85)
nd/rec GBM 5/3/2	Individual TAA mRNAs	TNFα, IL-1β, IL-6, PGE2	i.d.	3–8×: 2–4 weeks of intervals			No severe AE; others: skin rash, fever	(86)
rec GBM 1/0/1	CMVpp65		i.d.	3× weekly 5 × 10^6			No grade 3/4; mild fever, lymphopenia (TMZ)	(87)

rec, recurrent; nd, newly diagnosed; HGG, high grade glioma (grade III and IV); EGFRvIII, epidermal growth factor receptor variant III; KLH, keyhole limpet hemocyanin; IL-13Rα2, interleukin-3 receptor α2; CSC, cancer stem cells; mRNA, messenger ribonucleic acid; CMV, cytomegalovirus; WT-1, Wilms' tumor 1; PB, peripheral blood; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; PGE2, prostaglandin E2; OK432, preparation of streptococcus pyrogenes; IFNγ, interferon-γ; MCM, monocyte-conditioned medium; IL-6, interleukin-6; IFNα, interferon-α; poly(IC), polyinosinic:polycytidylic acid; LPS, lipopolysaccharide; i.d., intradermal; s.c., subcutaneous; i.t., intratumoral; i.n., intranodal; i.v., intravenous; SD, stable disease; PR, partial response; MR, mixed response; CR, complete remission; OS, overall survival; PFS, progression-free survival; DTH, delayed-type hypersensitivity.

[1]EphA2, IL13Rα2, YKL-40, GP100.

[2]WT-1, HER2, MAGE-A3, MAGE-A1, GP100, KLH.

[3]HER2, TRP-2, GP100, MAGE-1, IL-13α2, AIM-2.

[4]TRP-2, GP100, HER-2/NEU, Survivin.

[5]MAGE-1, AIM-2, HER2, TRP-2, GP100, IL13Ra2.

[6]WT-1, HER2, MAGE-A3, MAGE-A1, GP100, (KLH).

[7]In all studies, monocyte-derived DC were used.

[8]OS/PFS calculated from data provided in the manuscript.

[9]Toxicities have not clearly been attributed to DC vaccination.

[10]IFNγ responses have been detected by ELISA, enzyme-linked immuno spot (ELISPOT) assay, intracytoplasmic staining and flow cytometry, or quantitative PCR (qPCR).

[11]Others include proliferative or cytotoxic responses towards targets, tetramer staining, and flow cytometry and increase in GM-CSF, TNFα, IL-2, and IL-17a secretion upon specific restimulation and in tetramer-staining cells.

[12]Manuscript did not discriminate between GBM and grade III tumors or newly diagnosed and recurrent GBM.

Most patients underwent cytoreductive surgery prior to DCV, but patients who were biopsied only or had no surgery at all underwent treatment as well. An association of survival and the extent of resection, which by itself is predictive for better survival (89), has also been reported for DCV (78), and a state of minimal residual disease has been indicated to be beneficial for vaccination therapy (44, 48, 65, 78). This may be due to a reduction of local immunosuppression, which correlates with the tumor size (90, 91), and the sheer number of fast growing tumor cells, which otherwise would have to be eliminated by the CTL. However, in another study, the extent of resection was not associated with survival (76), so a more detailed comparison of the absolute residual tumor volume and, in particular, the composition of the tumor [e.g., the contribution of an immunosuppressive tumor microenvironment (TME)] is required.

Therapies concomitant to DCV included radio- and chemotherapy, mainly with TMZ (4); but in several trials, DCV has been used as the sole treatment. There may be advantages but also disadvantages for vaccination in the context of TMZ chemotherapy (see also the section *Dendritic Cell Dose, Vaccination Schedule, and Route of Application*).

In the previous trials, mainly patients with newly diagnosed or recurrent GBM have been treated with DCV, but in several studies, grade III tumors were also included (**Table 1**). It is unknown whether there is a difference in the responsiveness of grade III and grade IV tumors to DCV; however, a trend for a higher immunological response rate in newly diagnosed patients has been reported (50), which may be due to the less heavy pretreatment of those patients.

Overall, DCV was well tolerated (**Table 1**). Severe side effects (≥grade 3) attributable to vaccination have not been observed except for one patient with gross residual tumor post-surgery, who suffered from peritumoral edema, which was controllable by glucocorticoids (41, 48). Other severe side effects have been consistent with either the respective concomitant therapies or disease progression. Frequently observed mild and easily controllable toxicities (≤grade 2), which may be attributable to DCV, are injection site reactions with itching, pain, erythema, induration, and lymph node swelling as well as flu-like symptoms, fever, fatigue, myalgia, headache, edema, and meningeal irritation, which, however, can also be observed in the course of other concomitant therapies or be due to the disease. Thus, overall toxicity of DCV therapy is limited. However, it has to be noted that Mitchel et al., who vaccinated a GBM patient with DC transfected with cytomegalovirus (CMV) phosphoprotein 65 (pp65) mRNA and applied the vaccines intradermally together with GM-CSF, reported induction of a type I hypersensitivity-like reaction with IgE, but also IgM and IgG antibodies against the GM-CSF (92), which however resolved when vaccination was continued without GM-CSF. On the one hand, these results document the potency of DCV to induce immune responses. On the other hand, however, they urge caution in using any type of protein supplement at higher doses injected together with the DC.

Induction of antigenic target-directed immune responses have been observed in the course of DCV (**Table 1**), with detection of IFNγ responses being most informative (50, 71, 77, 82, 83, 85), but antitumoral cytotoxic responses (35, 42–44) and an increase in tetramer positive cytotoxic T cells (43, 56, 61, 67, 72–74) have been reported as well.

Several studies identified immunological responders based on antigenic-target directed delayed-type hypersensitivity (DTH) reactions, IFNγ responses, or cytotoxic responses, which increased in the course of vaccination and reported longer survival times for responders (44, 45, 50, 55, 66, 77, 81, 82). Indeed, in the study by Wheeler et al., immunological responders (IFNγ qPCR) had a significantly longer OS (599 *vs.* 401 days) and time to progression (260 *vs.* 146 days), and the 2-year OS rates compared favorably (56% *vs.* 8%) for the responders compared with the non-responders (50). Moreover, long-term survivors with durable IFNγ responses have been identified (71). However, although there are reports of an association of detectable antitumoral immune responses and better clinical outcome, this is not true for all studies (67, 75). Indeed, there appears to be no strong correlation between detection of systemic antitumoral immune responses and clinical outcome, which may be due either to the parameters tested or to a failure of the systemically detectable response in reaching the brain and effectively killing the tumor cells.

Survival of vaccinated patients compared favorably with matched or historic controls (35, 44, 45, 51, 54, 57, 68, 71, 85, 86). For newly diagnosed patients, mOS ranged from 15 to 41.4 months, and the progression-free survival (PFS) ranged from 6 to 25.3 months (**Table 1**).

Meanwhile, several controlled studies have been published, six of them randomized (60, 62, 76, 79, 81, 83), whereas Batich et al. summarized the data of three previous similar trials they conducted (84) (for details, see **Table 1**).

For a phase III trial on 331 newly diagnosed GBM patients (232 in the DCV group and 99 in the control group), Liau et al. described long-term survivors and a mOS of all patients of 23.1 months. Unfortunately, however, they did not yet report conclusive data on the outcome of the study.

In two randomized phase II trials with 34 (60) and 25 (62) newly diagnosed GBM patients, mOS (31.9 and 17 months) of the vaccination groups was significantly improved compared with that of the respective control groups (15 and 10.5 months).

In another randomized phase II trial with 41 newly diagnosed and recurrent GBM patients (81), Yao et al. reported that DCV significantly prolonged mOS (13.7 *vs.* 10.7 months).

In two randomized phase II trials with 76 (76) and 124 newly diagnosed GBM patients (83), no significant differences in mOS (18.8 *vs.* 18.9 months and 17 vs. 15 months, respectively) between patients in the DCV and control groups were observed, although Wen et al. reported a significantly improved PFS for the vaccinated patients (11.2 *vs.* 9 months).

Batich et al. (84) merged their data on DCV with CMVpp65 mRNA transfected DC of newly diagnosed GBM patients (71, 74), either admixed with GM-CSF (11 patients) or tetanus-diphtheria toxoid (Td; six patients) conditioning of the vaccination site. They reported a mOS of vaccinated patients of 41.1 (GM-CSF) and 41.4 months (Td) compared with 18.5

months for control patients receiving unpulsed DC (six patients). Moreover, they describe long-term survival rates at 5 years of 36.4% and 33.3%.

Thus, even from these controlled trials, which revealed mixed results, it is still difficult to draw a conclusion as to the efficacy of DCV in GBM. Since markers of immunosuppression such as the $PD\text{-}1^+$:$CD8^+$ ratio (78), the presence of regulatory T cells (Treg) or CTLA-4 expression pre- and post-vaccination (67, 93), and an immunocapability score (77) are associated with survival after DCV, combining DCV with tools interfering with immunosuppression seems to be called for to improve efficacy. Moreover, to improve DCV efficacy may also require further optimization of the DC vaccine in respect to target antigen selection, preparation of the cells, the integration of DCV into other treatment regimens, and dosing and scheduling of the vaccination(s).

ANTIGENIC TARGET

Efficacy of DCV depends on the presence of TAA or so-called neoantigens in the individual tumor, which allow specific recognition and killing of the tumor cells. The overall mutational load—the frequency of neoantigens—of this tumor entity is low, and the majority of GBM (>85%) contain only up to 10 mutations/1.4 Mb, except for patients with recurrent tumor after TMZ chemotherapy, which increases the mutational load [(94); for review, see (95)]. Nevertheless, multiple TAA have been identified for GBM (96–99).

In previous DCV studies, tumor lysates, apoptotic bodies of tumor cells, irradiated tumor cells, tumor mRNA, and fusions of tumor cells and DC as well as peptides eluted from the surface of tumor cells have been used as whole-tumor cell sources of TAA (**Table 1**). They are produced from the patient's own tumor obtained from surgery and can also be derived from subsets such as cancer stem cells (68, 81), which could provide a therapeutic advantage (28, 29, 100). Whole-tumor cell sources of TAA most likely will contain multiple TAA, which are present in the individual tumor of a patient (including the various cellular subsets within the tumor), i.e., the patient's full antigenic repertoire, ensuring antigenic diversity, thereby reducing the risk of escape of TAA-loss variants (101). Since the respective proteins are endogenously processed in the DC, in contrast to e.g., synthetic HLA class I restricted peptides, presentation on HLA class I and II molecules is possible and independent of the HLA type of the patient, thereby allowing induction of CTL as well as T_H responses at the same time, which is a prerequisite for the development of an efficient CTL response. At least for tumor lysates, presentation/cross-presentation *via* HLA class I and II molecules of lysate-derived peptides has been shown (102). The observation of CTL responses after DCV is further evidence that the material taken up by the DC is indeed cross-presented *via* HLA class I (**Table 1**). Furthermore, whole-tumor cell sources of TAA, which have not been processed or cultured extensively, may provide the so far unknown necessary signals, allowing the DC to guide effector T cells to the brain (103).

TAA represent only a small fraction of proteins; therefore, a low tumor content of the tumor sample used for preparation of the TAA would even further reduce the TAA concentration. While this may not pose a higher risk to the patients, it may compromise efficacy of the vaccine. Therefore, a high tumor cell content of the sample has to be ensured, a task that will benefit from fluorescence-guided surgery, which allows intraoperative identification of the "solid" part of the tumor (63). Nevertheless, an estimate of the tumor cell content should always be obtained and reported when publishing DCV data, to establish values relevant for efficacy, which are currently unknown. When using protein solutions, such as lysates, the protein concentration used to load the DC should also be determined and reported. Despite the abundant presence of normal self-antigens in whole-tumor cell sources of TAA, induction of autoimmunity or other severe side effects attributable to its use for vaccine production have not been reported (**Table 1**). However, when using whole-tumor cell sources of TAA, it has to be kept in mind that they may inhibit vaccine production, i.e., DC differentiation and maturation, or modify the function of the resulting DC, because of the presence of immunosuppressive factors produced by the tumor cells (104). Moreover, efficacy appears not to be based alone on the source of TAA, but also on how it is processed. Gark et al. reported that induction of immunogenic cell death prior to DC-loading increases survival of animals substantially and shifts responses in the brain towards T_H1/CTL/T_H17 (105).

As an alternative to whole-tumor cell sources of TAA, molecularly defined TAA such as specific peptides, proteins, and DC transfected with the respective target antigen mRNA have been used for DCV of GBM (**Table 1**). Molecularly defined TAA represent a more standardized, consistent, and reproducible source of TAA and offer the advantage of higher available target antigen concentrations and lower background, and target-specific responses can be easily monitored. They can even be produced personalized (86). Nevertheless, multiple molecularly defined TAA should be used to reduce the risk of TAA-loss variants escaping immune control (101).

It is currently unknown whether whole-tumor cell sources of TAA or molecularly defined TAA (and which ones) are superior in inducing antitumoral immune responses and more beneficial clinically in GBM. Irrespective of the source of TAA, induction of antitumoral T-cell responses by DCV has been reported in previous studies (**Table 1**). The controlled studies, which documented a clinical benefit, used tumor lysates, thus a whole-tumor cell source of TAA (60, 62, 81), as well as CMVpp65 transfected DC, thus a molecularly defined TAA (84). Similarly, in the controlled studies that did not report a clinical benefit, either tumor lysates (76) or a set of six defined peptides (83) was used.

When peptide and tumor lysate-loaded DC were compared in animal models, superior efficacy has been reported for lysate-loaded DC (106). Moreover, Neller et al. concluded from the analysis of 173 published immunotherapy trials on various tumor entities, including melanoma, renal cell and hepatocellular carcinomas, and lung, prostate, breast, colorectal, cervical, pancreatic, and ovarian cancers a higher

objective response rate (8.1% *vs.* 3.6%) when whole-tumor cell TAA were used compared with molecularly defined TAA (107). Thus, there appears to be an advantage of whole-tumor cell sources of TAA. However, particularly results from DCV against CMVpp65, a target that may be present in the majority of GBM patients and appears not to be expressed in normal brain cells (108, 109), generated convincing results for the efficacy of a molecularly defined TAA (71, 74, 84).

DENDRITIC CELL VACCINE

In 1994, Sallusto et al. reported the generation of immature DC with high antigen uptake activity from blood monocytes using the cytokines GM-CSF and IL-4, which then could be matured by an additional culture period with TNFα, resulting in cells with potent T-cell stimulatory activity (110). These so-called monocyte-derived DC are potent stimulators of naïve $CD4^+$ T_H cells and can polarize T_H-cell responses towards T_H1, cross-present antigens, and activate $CD8^+$ CTL [for review, see (14)]. Yet their activity depends on the maturation or activating stimulus.

Although there are now techniques to enrich the various rare blood DC populations, which may also be promising candidates for DCV (111), monocyte-derived DC have been used in all DCV trials in GBM to date (**Table 1**). Monocytes were enriched from either peripheral blood or leukapheresis products by adherence, immunomagnetic selection of $CD14^+$ cells, immunomagnetic depletion of B-cells and T cells, or elutriation. Depending on the enrichment procedure, monocyte purity varied, with $CD14^+$ selection yielding the highest purity. Higher monocyte purity may result in more stable culture conditions, reduces modulating effects of contaminating cells, and yields higher-purity DC preparations. Overall, production of the vaccine is more reproducible and results in a more homogenous cell population.

Typically, monocytes were cultured with GM-CSF and IL-4 in a first culture phase, generating immature DC within ~6 days. The antigen-uptake activity of immature DC is well developed. Therefore, they have been used for antigen loading, when tumor lysates, apoptotic bodies of tumor cells, irradiated tumor cells, fusions of tumor cells and DC, proteins, and mRNA transfection were used as source of TAA, whereas peptide pulsing was performed mainly with mature DC.

Immature DC are poor stimulators of T cells and can even induce tolerance. Only when they are activated, e.g., by proinflammatory signals, do they develop into mature DC (14, 111). Originally, TNFα has been used as maturation factor (110). In 1997, Jonuleit et al. described a more potent maturation stimulus, a cytokine cocktail containing IL-1β, IL-6, and TNFα together with PGE2 (112). This cocktail and other combinations of these factors with or without factors such as type I and II interferons, lipopolysaccharide, and toll-like receptor ligands [e.g., polyinosinic:polycytidylic acid (poly(IC))] have been used in the clinical studies summarized here (for details, see **Table 1**).

The optimal maturation stimulus is still a matter of debate and since immature DC sense, integrate, and translate environmental changes into signals to the T cells, differences in medium, cell density, the frequency of dead cells in cultures, etc., may result in different outcomes in regard to target cell function. Moreover, the same factors can have beneficial as well as adverse effects. For example, the combination of lipopolysaccharide and IFNγ induces semi-mature DC, which produce IL-12 and induce CTL responses (113), but it also appears to initiate an immunosuppressive program with the induction of indoleamine-2,3-dioxygenase [IDO; (114)]. Similarly, PGE2, which is part of a potent cytokine cocktail inducing DC maturation (112) and improves the migratory response of DC (115), can also induce IDO (116). In addition, the effects of the maturation factors can be further modulated by the source and processing of the TAA (104, 105). Thus, outcome of DC maturation is difficult to generalize. Therefore, the functional properties of the cells have to be determined for the respective conditions used in each manufacturing process.

Mature DC can be distinguished from immature DC by their expression of the surface molecules CD83 (117) and CD25 (118), whereas other markers such as CD40, CD80, CD86, and HLA-DR may differ only in expression density (118). Indeed, high-density expression of CD80 appears to be of utmost importance because it interacts in-cis with PD-L1, thereby blocking PD-L1 binding to PD-1 on T cells and inhibition of T-cell activation (119). However, identification of "mature" DC may be even more complex. Previously, a population of mregDC (mature DC enriched in immunoregulatory molecules) has been identified, which co-expresses maturation markers including CD83, CD40, CD80, CD86, and RelB together with immunoregulatory genes such as PD-L1, Pdcd1lg2, CD200, Fas, Socs1, Socs2, and Aldh1a2 (120).

Overall, mature DC for immunotherapy should 1) have potent T_H-cell and CTL stimulatory activity; 2) polarize responses towards T_H1, which is required for efficient induction of effector CTL (121, 122); 3) have to imprint effector T cells for brain tumor homing, which may require induction of VLA-4 (α4/β1 integrin), which appears to be the main integrin for lymphocyte trafficking to the brain (123); 4) express CCR7, which is required for lymph node homing (124, 125); 5) be phenotypically stable upon withdrawal of cytokines (126) to prevent re-differentiation towards immature (and possibly tolerogenic) DC after administration; 6) be resistant to immunosuppressive cytokines like TGF-β (127); and 7) not induce tolerance. Particularly, the induction of target antigen-specific tolerance, which has been reported for immature and semi-mature DC, but not fully mature DC would be detrimental to the intended induction of antitumoral immune responses (128). Indeed, de Vries and colleagues showed that TAA-loaded mature DC, but not immature DC, induce immunological responses in melanoma patients (129), and even more important, Dhodapkar et al. documented a decline of the influenza matrix peptide-specific T-cell response in healthy individuals after vaccination with matrix peptide-loaded immature DC (130).

In GBM, several clinical trials used immature DC as vaccines (**Table 1**). Somewhat unexpectedly, immunological responses as well as beneficial effects on survival have been reported, although

Yamanaka et al., who used mature as well as immature DC in their study, reported a trend towards a better outcome in GBM patients vaccinated with the mature DC (45). Two of four controlled trials reporting a clinical benefit used immature DC (60, 81), whereas in the remaining two trials, DC were matured with TNFα, IL-1β, and PGE2 (62) or TNFα, IL-1β, IL-6, and PGE2 (84). Interestingly, both controlled trials that did not document clinical efficacy (76, 83) used lipopolysaccharide + IFNγ for maturation, thus a factor combination that also results in the induction of IDO (114). In conclusion, currently, the most potent vaccine for DCV of GBM has not been identified, and even the use of immature DC cannot be excluded, although there are strong arguments for the use of mature DC.

DENDRITIC CELL DOSE, VACCINATION SCHEDULE, AND ROUTE OF APPLICATION

The minimum DC dose reported to elicit T-cell responses in healthy individuals is 2×10^6 DC/vaccine (16). A wide range of DC doses have been used in GBM-DCV trials ($0.25–100 \times 10^6$ DC/individual vaccine, **Table 1**). In four controlled studies, which reported a significant survival benefit for vaccinated patients, doses of $2–4 \times 10^6$ (81), 6×10^6 (62), 20×10^6 (84), and $20–50 \times 10^6$ (60) DC/vaccine were used, while doses of $1–5 \times 10^6$ (76) and 11×10^6 DC/vaccine (83), i.e., in a comparable range, resulted in no clinical benefit in two other controlled trials. In several studies, immunological responders were identified based on an increase in IFNγ (qPCR, ELISPOT) after DCV, and immunological responsiveness was positively associated with survival. DC doses ranged from 1 to 50×10^6 DC (50, 71, 77, 85). No correlation with DC dose has been described for either clinical outcome or immunological responsiveness, and a dose–response relationship with an optimal dose cannot yet be defined. Because dose-limiting toxicity has not been reached in previous studies, doses tend to be maximized based on the number of cells available from the production process and the vaccination scheme. In a dose-escalating study by Prins et al. using 1, 5, and 10×10^6 DC/vaccine, no association was found between increasing DC dose and toxicity or immunologic response, but longer survival (though not statistically significant) was observed in those patients receiving the lowest DC dose (57). Although only a fraction of the injected DC reaches the lymph nodes (131, 132), this may still be far more than, e.g., in the case of infections and could actually be too much. However, Mitchel et al., who used fairly high DC doses/vaccine (20×10^6), reported an improved survival of patients, when the efficiency of migration of DC to the lymph nodes was enhanced (71). Moreover, intranodal application, which may allow to directly deliver even higher DC numbers to the lymph nodes, resulted in increased IFNγ responses after vaccination (55, 56, 77), although Buchroithner et al. could not document a survival benefit in a controlled trial using this approach (76). However, it has to be kept in mind that DC vaccines are likely to differ in potency, e.g., immature as well as mature DC have been used, and the vaccination schedule and route of application, as well as many other parameters, also influence efficacy, so an optimal DC dose is difficult to define and may need to be determined on a case-by-case basis for each vaccination strategy, yet it has the potential to improve efficacy.

Multiple vaccinations were given per patient, mainly 3–10, but also up to 23 (**Table 1**). Thus, many studies used a prime and boost vaccination approach. Currently, it is not clear whether multiple vaccinations improve the outcome. Jouanneau et al. showed in a GL26 orthotopic tumor model that multiple injections of TAA-loaded DC did not further improve the outcome, whereas a lysate boost resulted in a significantly prolonged survival and was associated with an increased CTL response and antibody formation, contributing to the therapeutic effect (133). Indeed, de Vleeschouwer et al. described a trend of prolonged PFS with a DCV strategy with lysate boosting, although the contribution of the lysate boosts remains unknown (48). In contrast, Okada et al. reported that DC boost vaccination further enhanced IFNγ responses (56) and Buchroithner et al. described a trend towards better survival in patients receiving more vaccines (76). Thus, the number of vaccinations and the use of DC vaccines or lysates (or any other target antigen such as e.g., peptides) for boost vaccination are parameters that influence efficacy and remain to be optimized.

Vaccines have been administered weekly, biweekly, or monthly, or in combinations thereof, frequently integrated into established treatment regimens of radiotherapy and chemotherapy. The standard of care for newly diagnosed GBM consisting of resection, and radiotherapy with concomitant and subsequent adjuvant TMZ chemotherapy (4) appears to offer several windows of opportunity for vaccination, allowing to retain the standard of care with proven efficacy while integrating DCV and potentially exploiting synergies between the two therapeutic approaches.

Immunosuppression, which is prominent in GBM (see below), correlates with tumor size, and surgical cytoreduction can at least partially restore immunological responsiveness (90, 91). Fluorescence-guided surgery (FGS) allows to increase the extent of resection safely, and radiologically complete resections could be performed in 65% of patients compared with 35% in a control group undergoing standard surgery (89) that was associated with improved survival. Thus, integrating FGS into a DCV approach (63, 75) may not only minimize residual tumor mass and thereby also immunosuppression, both of which are beneficial for immunotherapy (44, 48), but because of the extended PFS, it may also prolong the time period available for T-cell responses to clear residual tumor cells before the tumor mass becomes too large again. Moreover, FGS ensures high tumor cell frequency in the tumor samples, because the vital "solid" part of the tumor can be identified intraoperatively (63), an advantage when whole tumor cell sources of TAA such as tumor lysates are used. The substantial increase in the extent of resection by FGS may also improve safety of DCV, because in a patient with gross residual tumor after standard surgery, a grade 4 peritumoral edema has been reported, which was considered to

be associated with DCV (41, 48). In addition, a maximal resection generally allows to wean glucocorticoids faster, which are perioperatively applied in GBM patients to reduce swelling in the brain—a possible advantage for DCV due to the immunosuppressive activities of the steroids. However, although glucocorticoids are potent inhibitors of T-cell immunity (134) and Keskin et al. reported induction of polyfunctional T cells after peptide vaccination only in those GBM patients that did not receive dexamethasone (135), its role during DCV is not entirely clear. Therefore, it should be used judiciously or excluded, until more data on dosing and timing of glucocorticoid use during DCV of GBM patients become available (136). Indeed, in four of the controlled trials (79, 81, 83, 84), the use of glucocorticoids was either excluded entirely or only minimal doses of 2–4 mg/day of dexamethasone were allowed.

Vaccination is either performed in the time period between radiochemotherapy and adjuvant TMZ or in the course of the adjuvant TMZ cycles around day 21 (137), because there appears to be a rationale to combine DCV with TMZ chemotherapy: 1) TMZ can improve immunological responsiveness (138–140), probably by reducing Treg (see below) (139, 141) and interfering with their recruitment to the tumor (142). 2) Although it frequently causes lymphopenia, the recovering lymphocyte compartment after chemotherapy has been shown to allow for efficient induction of antitumoral responses (143–145). 3) Dying tumor cells after radiochemotherapy or chemotherapy lead to a release of tumor antigens, which could enhance homing of tumor-specific effector CTL to the brain tumor after luminal presentation of the target peptides on HLA class I molecules on the cerebral endothelium (146). However, effects appear to depend on TMZ dose; e.g., lower but not higher TMZ doses were shown to deplete Treg (141), whereas myeloablative but not non-myeloablative doses enhanced responses to a peptide vaccine (147). In addition, results from Pellegatta et al. indicate that adjuvant TMZ may deplete $CD8^+$ T cells previously expanded by DCV, because in contrast to NK cells, they fail to express the multidrug resistance transporter protein ABCC3 (80). A decline in responding cells after adjuvant TMZ has also been described by Batich et al. (84). Moreover, is has been shown that DCV only in the absence of TMZ, although with additional conditioning of the injection site with tetanus toxoid, results in the generation of T effector memory cells producing IFNγ, which is positively associated with survival (82). These results would argue against combining DCV and TMZ chemotherapy. Because all controlled DCV trials in GBM used TMZ in both arms, currently, it cannot be determined whether or not it affects efficacy (60, 62, 76, 81, 83, 84).

Effective induction of antitumoral T-cell immunity requires the DC to reach the T-cell areas of lymph nodes. Although the cervical nodes (148–151) or the nasopharynx-associated lymphoid tissue (152) serve as lymph node stations of brain immune responses, imprinting the brain homing phenotype of effector T cells is a function of the DC rather than that of a distinct lymph node. Therefore, effective responses targeting antigens in the brain can also be initiated in other lymph nodes besides the cervical nodes or the nasopharynx-associated lymphoid tissue (103).

Depending on the route of application, DC can be detected in different organs. Intravenous application results in a rapid enrichment in the liver, lungs, and kidneys but is the highest in the spleen, whereas after subcutaneous application, there is a marked accumulation of DC in the draining lymph nodes, with a preferential paracortical localization in the T-cell areas (153). When intradermal application is used instead, even more DC reach the T-cell areas of the lymph nodes in mice (154) as well as in humans (155), with only mature but not immature DC efficiently migrating to the lymph nodes (156). This is probably due to the expression of the CCR7 chemokine receptor on the mature DC (118, 124, 125) and the responsiveness of the cells to the chemokines CCL19 and CCL21, which are expressed constitutively by peripheral lymphatic endothelial cells and lymph node stromal cells (157).

DC can already be detected in the lymph nodes 30 min after injection, there is a maximum after 48 h, and they appear to persist for up to 14 days (158–160). Only ~5% of injected DC may reach the lymph nodes, which appears to be sufficient for effective induction of antitumoral immune responses (131, 132), although substantially higher values have been reported as well (160). A large fraction of DC remains at the injection site, rapidly becomes apoptotic, and is cleared by $CD163^+$ macrophages (131). However, it is possible to augment DC migration to the lymph nodes by preconditioning the application site with a potent recall antigen such as tetanus/diphtheria toxoid, associated with improved survival of patients (71).

The life span of DC in the lymph nodes is limited to a few days (161, 162), and they may be removed by apoptosis (163, 164) and phagocytic clearance by macrophages (165). However, endogenous DC in skin and lymph nodes may prolong antigen presentation beyond the life span of the injected DC (166).

Overall, there appears to be an advantage of intradermal application of vaccines, and indeed, most studies have used it (**Table 1**). Whether the higher DC numbers delivered directly to the lymph nodes by intranodal application (55, 56, 76) are even more effective remains to be determined.

There is also the possibility of intratumoral application of DC. Pellegatta et al. have documented in an orthotopic GL261 glioma model that the efficacy of intratumoral application of GL261 lysate-loaded DC is lower than that of subcutaneous application, but that the combination of both procedures significantly improves survival (167). Since intratumorally administered DC remain in the brain parenchyma and were not detected in the cervical lymph nodes, a different mechanism than for the subcutaneously administered DC appears to be responsible for improved survival. Whether they contribute to the final maturation and shaping of the effector T-cell response (168) by acting as tissue inflammatory DC or reduce tumor cell growth in this model because of their production of TNFα (167) remains to be determined. Intratumoral application has not yet been studied in clinical trials in GBM.

IMMUNOSUPPRESSION AND IMMUNE CHECKPOINT REGULATION

A major obstacle to the therapeutic vaccination of GBM with DC is that the antitumoral immune response must be elicited in the context of immunosuppression. Humoral and cell contact-dependent mechanisms originating not only from the tumor cells themselves (including intrinsic mechanisms of immune evasion) (169) but also from the cells of the TME, such as Treg, tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC), can inhibit antitumor immunity (**Figure 1**). Moreover, immune checkpoints—control mechanisms that limit and thereby prevent excessive immune responses—may be activated in the TME, also resulting in inefficient responses and T-cell dysfunction. Whether DCV by itself can tip the balance towards immunity is unclear, but efficacy may require the combination with additional therapeutic strategies (**Figure 2**) to overcome the adverse effects of immunosuppression and immune checkpoint regulation.

Regulatory T Cells

Treg are a distinct immunosuppressive T-cell subpopulation, which contribute to maintaining immunological tolerance, limiting excessive immune responses, and promoting homeostasis and tissue regeneration. They either develop in the thymus (thymic (t)Treg) as a distinct T-cell lineage or differentiate in the periphery (peripheral (p)Treg) from naïve T cells [reviewed in (170, 171)]. Naïve/resting Treg reside mainly in the blood and secondary lymphoid organs. They can be identified as $CD3^+/CD4^+/CD25^{low}/CD127^{-/low}$ T cells, which express the transcription factor FoxP3 (172–176), although FoxP3 may also be transiently expressed at low levels in the course of activation of human conventional $CD4^+$ and $CD8^+$ T cells (177). They further express CD45RA, CD62L, and CCR7 (CD197), whereas expression of CTLA-4 (CD152) and CD45R0 is absent on the naïve/resting Treg. Upon T-cell receptor stimulation, they differentiate into highly proliferative and suppressive effector Treg, which are characterized by a $CD3^+/CD4^+/CD25^{high}/CD127^{-/low}/FoxP3^{high}/CD45RA^-/CD45R0^+/$

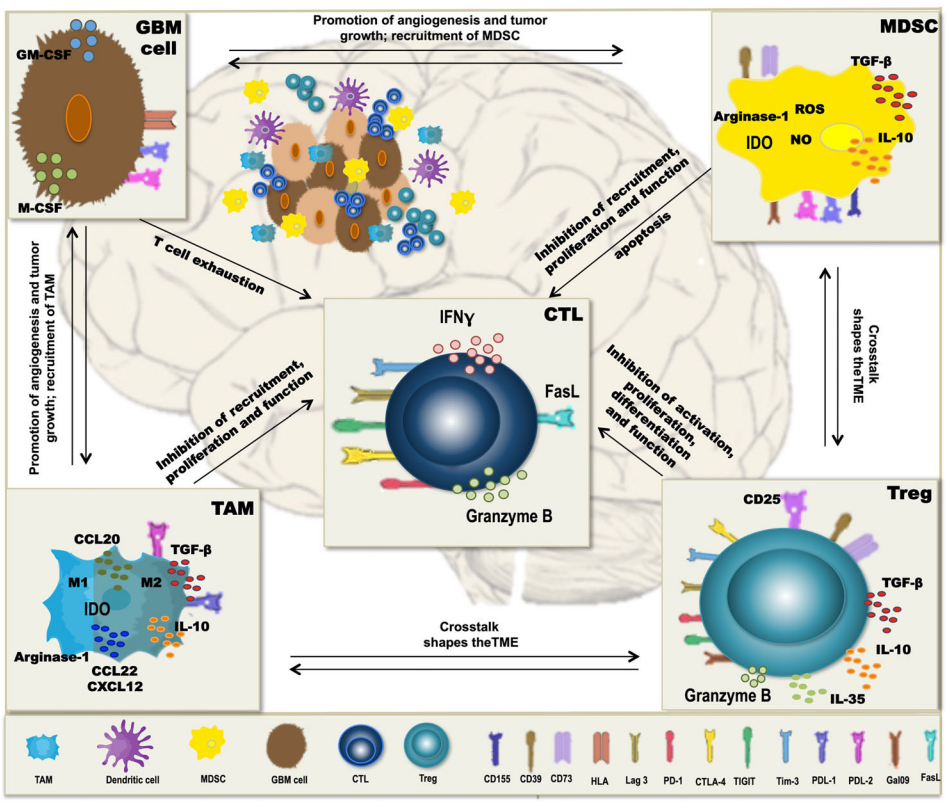

FIGURE 1 | Mechanisms of immunosuppression in glioblastoma (GBM). In GBM, tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and regulatory T cells (Treg) form a potent immunosuppressive tumor microenvironment (TME), which inhibits antitumor immunity and thereby interferes with dendritic cell vaccination (DCV). Besides the intrinsic immune escape mechanisms of the tumor cells, immune checkpoint molecules, like PD-L1, PD-L2, Tim-3, Lag-3, CD155, and galectin-9 that normally control the extent of immune responses, are expressed on the immunosuppressive cells of the TME, contributing to T-cell dysfunction and subsequently inefficient antitumoral immune responses. Cells of the TME secrete cytokines such as TGF-β, IL-10, and IL-35 and the chemokines CCL20, CCL22, and CXCL12, which inhibit T-cell proliferation and function and contribute to a crosstalk between the different TME cell types, thereby further enhancing immunosuppression. Additional mechanisms include the activity of indoleamine-2,3-dioxigenase (IDO) and arginase-1 as well as production of reactive oxygen species (ROS) and nitric oxide (NO), all interfering with a proper differentiation, expansion, and function of effector T cells.

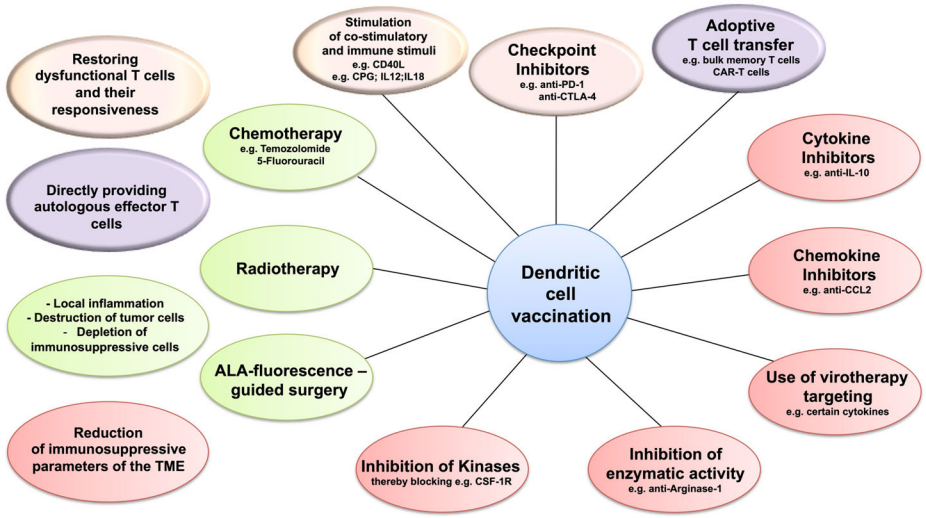

FIGURE 2 | Dendritic cell vaccination (DCV) and targeting the immunosuppression in the tumor microenvironment (TME). Combining DCV with therapies targeting the three immunosuppressive cell populations of the TME—regulatory T cells (Treg), tumor-associated macrophages (TAM), or myeloid-derived suppressor cells (MDSC)—might improve efficacy. Potential target strategies include restoring the responsiveness of the dysfunctional T cells (pink), applying effector T cells by adoptive transfer (purple), depleting immunosuppressive cells, and modulating the inflammatory conditions in the TME (green) and blocking the mechanisms of immunosuppression (red).

$CCR7^-/CD62L^-/CTLA\text{-}4^+$ immunophenotype [reviewed in (170, 178)], with delineation of cells further improved by the presence of CD15s on effector Treg, but not conventional effector T cells (179). Depending on the stimulatory context and the affinity of T-cell receptor recognition, there is induction of chemokine receptors (e.g., CCR2, CCR4, and CXCR3) and adhesion molecules (e.g., lymphocyte function-associated antigen-1 (LFA-1), integrin-α4, and integrin-β1), which guide them to their target sites, as well as of effector molecules, which mediate selective mechanisms of immunosuppression. Indeed, effector Treg can upregulate transcription factors associated with distinct T_H-effector phenotypes, allowing suppression to be tailored to the respective polarized responses, and it may be further modulated by the local microenvironment at the target site (170, 178, 180–182).

Multiple mechanisms contribute to effector Treg function:

1. They secrete the immunosuppressive cytokines IL-10, TGF-β, and IL-35, which inhibit T-cell activation and proliferation either directly or by suppressing the stimulatory activity of DC, and contribute to the formation of tolerogenic DC and the generation of additional Treg (183–186). These effects are further enhanced by cytokine-mediated crosstalk between the immunosuppressive cells of the TME, e.g., TGF-β stimulates MDSC proliferation and suppressive activity (187). Moreover, TGF-β downregulates intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on blood vessels and thus inhibits conventional T-cell infiltration into the TME (188).
2. Treg release granzyme and perforin, which induce apoptosis of effector T cells (189, 190). Moreover, $CD4^+$ T_H-effector cells expressing death receptor 5 (DR5) can also be killed by Treg expressing the corresponding ligand TNF-related apoptosis-inducing ligand (TRAIL) (191).
3. Due to the high-density expression of high-affinity IL-2 receptors (CD25/CD122/CD132) on Treg, they act as an IL-2 sink and deprive conventional T cells of IL-2, which suppresses their expansion and differentiation to effector cells and may cause effector T cells to become anergic or apoptotic (192).
4. Treg can release adenosine nucleosides. They express CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5′-nucleotidase), which together convert adenosine triphosphate to adenosine, with extracellular adenosine inhibiting DC antigen presentation as well as proliferation and cytokine secretion of activated T cells through the A2A receptor (193–195). It further promotes differentiation and proliferation of Treg, expansion of MDSC, and polarization of M2 macrophages [reviewed in (196)]. Treg also contain high levels of cyclic adenosine monophosphate, which they can transfer into conventional T cells *via* gap junctions, thereby inhibiting their proliferation and IL-2 production upon activation (197).
5. Effector Treg express several surface molecules, which interfere with the activation, proliferation, differentiation, and effector function of conventional T cells. CTLA-4 is an immune checkpoint regulator. It binds to CD80/CD86 on DC with higher affinity than CD28 on conventional T cells, leading to anergy, apoptosis, or even the conversion of the activated conventional T cells into Treg, due to the absence of the co-stimulatory signal. However, CTLA-4 not only competes with CD28 for CD80/CD86 binding but also depletes them from the surface of DC by transendocytosis, thereby further preventing

co-stimulation (198, 199). Moreover, it induces IDO in DC and causes their conversion to tolerogenic DC. IDO is an enzyme that degrades the essential amino acid tryptophan to kynurenine. The resulting local tryptophan depletion as well as the interaction of kynurenine with the aryl hydrocarbon receptor prevents T-cell proliferation and can induce differentiation of $CD4^+$ T cells into Treg (200–202). Other immune checkpoint regulators including PD-1 and its ligand PD-L1, lymphocyte-activation gene 3 (Lag-3), T-cell immunoglobulin mucin-3 (Tim-3), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) can also be expressed on Treg and contribute to immunosuppression (203–208) [reviewed in (209, 210)].

In various solid tumors, there is a high frequency of tumor-infiltrating effector Treg and particularly a high Treg : $CD8^+$ T-cell ratio, which negatively correlate with prognosis (211), indicating that there is a naturally occurring antitumoral immune response. Treg cannot be detected in normal brain, and they are rare in low-grade brain tumors, yet despite lymphopenia, Treg frequencies are increased in the TME as well as in the blood of GBM patients (212–215). Frequencies may vary by GBM subtype, with the $IDH^{wildtype}$ (Iso-citrate dehydrogenase) and mesenchymal subtypes having higher Treg frequencies than $IDH^{mutated}$ (216) and proneural and classical GBM subtypes, respectively (217). It has been reported that there is an age-dependent increase in the Treg : $CD8^+$ ratio, with a maximal increase in the 60–69 years age group, which coincides with the median age (64 years) of GBM patients at diagnosis (218). Nevertheless, the association of Treg frequency with prognosis is moderate at the most (188, 214, 215, 219), in contrast to $CD4^+$ and $CD8^+$ effector T-cell infiltrates, which are positively associated with survival (188). However, association appears to require the concomitant presence of a low immunosuppressive signature (220). Indeed, a negative association of the Treg : $CD8^+$ T cell ratio with survival has been reported (221), which could indicate that there are patients with natural antitumoral immunity and that only in these patients are Treg associated with poor prognosis.

In mouse models of GBM, a time-dependent accumulation of Treg after implantation of tumor cells has been described (222–224). Interestingly, Treg numbers increased first in blood and later, but still in the asymptomatic phase, in the tumor tissue (223, 224). Thus, Treg appear to be recruited to the tumor already in an early phase, when tumor cell numbers are still low and are not a consequence of the immune system simply being overwhelmed by the tumor mass at a later phase of tumor development. The chemokines CCL2 and CCL22, which are produced by brain cells as well as GBM cells and cells of the TME, appear to be responsible for the recruitment of the $CCR4^+$ Treg to the TME of GBM (142, 215, 225).

Treg-mediated immunosuppression has been targeted to enhance the efficacy of natural or induced antitumoral immunity mainly in preclinical models of GBM. This includes depletion of Treg by anti-CD25 antibodies (145, 222, 226, 227), interference with their immunosuppressive activity, e.g., by blocking surface molecules such as CTLA-4 (222, 228, 229), TIGIT (230), Tim-3 (231), and PD-1 (229, 230, 232) or enzymes like ecto-5′-nucleotidase (CD73) (233) and IDO (229), or by preventing the accumulation of Treg in the TME by blocking CCR4-mediated migration (225). Irrespective of the approach, these studies could document a beneficial effect, with increased survival and reduction of tumor burden associated with restoration of antitumoral immunity, particularly when different strategies were combined.

However, specific inhibition of Treg or of their functional activity may not be required. Higher doses of non-fractionated radiotherapy (234) and chemotherapy with low-dose TMZ (141) or cyclophosphamide (235) have also been reported to reduce Treg, although it is unclear whether the long-lasting lymphopenia induced by the standard concomitant radiochemotherapy of GBM patients is beneficial for antitumoral immunity (144, 145, 236). Moreover, immunogenic stimuli such as CPG oligonucleotides (237, 238), virotherapy (239, 240), or the use of agonistic antibodies specific for co-stimulatory receptors such as CD40 (241) and OX40 [CD134; (242)] may be sufficient to tip the balance towards antitumoral immunity, associated with a reduction in Treg. Another promising approach is to modulate the metabolism of Treg, which appears to be tightly linked to their survival and function in the TME [reviewed in (243)]. Whether combinations with therapeutic approaches targeting Treg or their function result in increased efficacy has not extensively been studied and requires further investigation. Curtin and colleagues reported that anti-CD25 depletion of Treg in combination with intratumoral delivery of an adenoviral vector expressing Fms-like tyrosine kinase 3 ligand and herpes simplex type 1-thymidine kinase inhibited clonal expansion of tumor antigen-specific T cells, T-cell dependent tumor regression, and long-term survival of animals (239), whereas a trend towards improved survival has been reported for the combination of radiotherapy and anti-IDO (244). Particularly, the timing of Treg depletion appears to be of utmost importance. Several of the target molecules (e.g., CD25 and CTLA-4) are not specific to Treg but are upregulated in the course of activation on conventional T cells as well. Similarly, although anti-PD1 treatment may enhance antitumoral immunity, at the same time, it may increase the suppressive activity of Treg (245). Thus, depletion has to be performed prior to the promotion of antitumoral immunity or other treatments, to avoid suppression of the antitumoral effector response (145, 239, 246). Moreover, long-term survival of glioma-bearing mice was only observed when the animals were treated by a combination of systemic and intracranial, but not by systemic anti-CD25 antibody treatment alone (247). Thus, depleting/blocking agents have either to be administered directly to the tumor in the brain or to be able to pass the blood–brain barrier efficiently. Local administration may also prevent uncontrolled inflammation and autoimmune phenomena due to the systemic elimination of Treg and thereby of an important control mechanism of immunity and tolerance.

Evidence for a prominent role of Treg for the efficacy of DCV comes from several studies: Driessen et al. identified a lower Treg frequency in cured rats compared with non-cured rats after DCV in a 9L gliosarcoma model (248). Fong et al. and Prins et al.

observed a prolonged survival in patients whose Treg decreased after vaccination with peptide-pulsed DC (67, 93), with higher Treg values before vaccination having been shown by Erhart et al. to be negatively associated with survival after vaccination with lysate-loaded DC (77). Although Batich et al. described long-term survivors after anti-CMVpp6 DCV, they observed increases in Treg that were, however, paralleled by an increase of the $CD8^+$:Treg ratio (74). Thus, the absolute numbers or frequencies of Treg by themselves may not be informative enough. Moreover, several studies reported a beneficial effect on survival when DCV was combined with anti-CD25 treatment, particularly when depletion was performed prior to vaccination (106, 227, 249). Thus, depletion of Treg or interference with their activity has the potential to improve the outcome of DCV.

Myeloid-Derived Suppressor Cells

MDSC are a heterogeneous population of immature myeloid cells with potent immunosuppressive activity. In the TME, they constantly interact with the infiltrating T cells, particularly CTL, and suppress their function (250, 251), thereby supporting tumor growth and progression (252). MDSC are divided into two general subsets: polymorphonuclear (PMN)-MDSC (CD11b +/$CD14^-$/CD15+ or $CD11b^+$/$CD14^-$/$CD66b^+$), similar to neutrophils, and monocytic MDSC ($CD11b^+$/$CD14^+$/HLA-DR^-/lo//$CD15^-$), similar to monocytes (250). However, while they are phenotypically similar to neutrophils and monocytes, they are functionally distinct (253, 254).

MDSC can be detected in cancer patients or during chronic inflammation (250, 251, 255), when persistent low-level stimulation of myelopoiesis results in the development of the immunosuppressive myeloid cells (256). They develop in the bone marrow and traffic into solid tumors, where they accumulate mediated by factors such as GM-CSF, M-CSF, G-CSF, VEGF, IFNγ, IL-6, and IL-4, which are secreted by the tumor cells themselves or other cells of the TME (257, 258).

MDSC, similar to Treg and in part overlapping (see above), use multiple mechanisms of immunosuppression [for review, see (259)], in particular including reactive oxygen species (ROS) and nitric oxide (NO)-dependent pathways. ROS and NO cause apoptosis of immune cells (260) and block entry of CTL into the tumor and responsiveness of T cells to HLA stimulation through the nitration of chemokines and T-cell receptors, respectively (261, 262). MDSC also inhibit extravasation of T cells by downregulating CD44 and CD164 (259) and lymph node re-circling through downregulation of CD62L on naïve T cells *via* expression of ADAM17 (263). Arginase 1 catabolizes the non-essential amino acid arginine and depletes it from the microenvironment. Similarly, there is a depletion of cysteine by consumption and sequestration and of tryptophan due to IDO activity. This lack of amino acids in the TME inhibits proliferation of activated T cells. Like Treg, MDSC can produce immunosuppressive cytokines such as IL-10 and TGF-β and extracellular adenosine through the enzymatic activity of CD39 and CD73. Moreover, they express ligands of immune checkpoint regulatory pathways such as PD-L1, PD-L2, CD155, and galectin-9, which dampen and suppress T-cell responses and may even cause T-cell apoptosis upon interaction with their receptors on T cells (259, 264). Moreover, there is extensive crosstalk between MDSC and Treg, further enhancing immunosuppression (265, 266).

In GBM, MDSC are a major immunosuppressive component of the TME (267). They are also significantly increased in the blood of patients, associated with a higher concentration of the MDSC-specific protein S100A8/9 and arginase activity in the serum (268). More recently, Alban et al. confirmed the presence of MDSC in the blood of GBM patients, while this cell population was completely absent in low-grade glioma patients and healthy individuals (269). In the tumor, MDSC can be found in close proximity to cancer stem cells, and their presence correlates negatively with OS (270). Analogous to Treg, MDSC appear to be recruited to the brain tumor in an early phase and have already been detected in premalignant lesions in a mouse model (271).

Early studies have confirmed the immunosuppressive activity of MDSC in glioma patients and shown that their depletion can restore the disturbed T-cell function (272). Depletion of MDSC with low-dose 5-fluorouracil (5-FU) resulted in prolonged survival in a glioma mouse model (270), and in a clinical study, it has been documented that metronomic capecitabine reduces MDSC, associated with an increase in T-cell infiltrates in the tumors (273). Moreover, their frequency correlates negatively with the response to immunotherapy as reviewed by Stewart and Smyth (274).

Thus, there is evidence for a prominent role of MDSC-mediated immunosuppression in GBM. It has been proposed that targeting MDSC might improve the response to other therapeutic approaches, particularly immunotherapy. The following main targeting strategies are considered: 1) depletion of MDSC, 2) blockage of their migration towards the tumor site, 3) abrogation of their immunosuppressive activity, and 4) pushing them into differentiation towards mature myeloid cells (258). Gao et al. have recently summarized all studies and agents targeting MDSC (275). Therefore, in the following section, only a few examples of the intervention with the immunosuppressive mechanisms of MDSC are presented.

The depletion of MDSC can either be achieved directly by low-dose chemotherapy with, e.g., 5-FU (270), capecitabine (273), or ibudilast (269), or indirectly by promoting their differentiation to either M1 macrophages by docetaxel (276) or towards DC with paclitaxel (277). Full maturation of MDSC can be induced by all-trans retinoic acid [ATRA; (278)]. Blocking of the CSF-1 receptor (CSF-1R) signaling *via* pexidartinib reduced MDSC as well as M2 macrophages (279), and STAT3 inhibitors can reduce the number of MDSC and interfere with their functional activity (271). Furthermore, because MDSC share several mechanisms of immunosuppression with Treg such as the utilization of checkpoint regulatory pathways, the same inhibiting strategies can be used (see *Regulatory T Cells* section).

Evidence for a role of MDSC for efficacy of DCV comes from a study on small cell lung cancer patients, who were vaccinated with p53-loaded DC together with ATRA treatment. DCV alone did not change the frequency of MDSC, which however was reduced twofold by ATRA. Moreover, the combination therapy

resulted in a significant increase of specific immune responses (280). In GBM, standard radiochemotherapy has been reported to reduce MDSC in a mouse model (281), but effects have not yet been assessed in humans in detail. Nevertheless, there may be a rationale for depletion of MDSC or interference with their activity together with DCV.

Tumor-Associated Macrophages

Macrophages display a high plasticity in response to microenvironmental cues, allowing them to acquire distinct phenotypes and perform diverse functions. In general, macrophages are divided into classically activated M1 macrophages and alternatively activated M2 macrophages (282, 283). Differentiation of monocytes to M1 macrophages is induced by GM-CSF and proinflammatory cytokines like IFNγ and TNFα, whereas M2 macrophages differentiate in the presence of M-CSF and anti-inflammatory stimuli (284–288). M2 macrophages can be further subdivided into M2a, M2b, and M2c macrophages (289, 290), with IL-4 and IL-13, immune complexes and TLR agonists, and IL-10, TGF-β, and glucocorticoids representing the major polarizing factors, respectively. Functionally, M1 macrophages are proinflammatory, promoting immunity, wound healing, and tissue regeneration (291, 292). In contrast, M2 macrophages are rather anti-inflammatory; and involvement in wound healing, tissue repair and T_H2 polarization (293–295), phagocytic and immunomodulatory activity (293, 296–299), and involvement in immunosuppression and angiogenesis (289, 300, 301) have been reported for the M2a, M2b, and M2c subtypes, respectively.

TAM are components of the TME. They frequently exhibit an M2-like phenotype and generally act pro-tumorally, and their presence is associated with poor prognosis. They originate from bone marrow-derived circulating monocytes and accumulate in the tumor due to the presence of M-CSF, GM-CSF, and CCL2 as well as other factors (302, 303). In the tumor, they differentiate into anti-inflammatory M2-like TAM by tumor and TME-derived factors like M-CSF, IL-4, IL-10, and TGF-β. Depending on the local conditions, they polarize towards the M2a, M2b, or M2c subtypes (296, 302, 304–306).

Besides other tumor promoting activities, the anti-inflammatory M2-like TAM promote immunosuppression. They express immunosuppressive cytokines such as TGF-β and IL-10, which not only inhibit T-cell proliferation and function but also contribute together with chemokines, such as CCL20, CCL22, and CXCL12, to an extensive crosstalk with Treg and MDSC, further enhancing immunosuppression (307–314). Moreover, similar to Treg and MDSC, depletion of amino acids (tryptophan and arginine) and expression of ligands of immune checkpoint regulatory pathways contribute to the immunosuppressive activity of TAM (201, 312, 315–320).

In GBM, TAM make up approximately 30%–50% of all cells in the TME (321–323). They are associated with poor prognosis and tumor progression (324–327). Besides infiltrating TAM, in GBM, there is a secondary population of brain-resident monocytic cells, the microglia, which may also be modulated in its activity by the tumor and other cells of the TME (328–331). Bone marrow-derived monocytes are recruited to the brain by cytokines and chemokines such as IL-1β and CCL2 (313, 332), where they mainly differentiate towards anti-inflammatory M2-like macrophages, particularly towards M2c (330, 333). Moreover, the polarization towards M2-like macrophages is an evolving process, which is highly dependent on a hypoxic TME. With increasing hypoxia, the cells polarize more and more towards the M2 subtype (334–336). In line with this observation, there appears to be an increase in M2-like macrophages in recurrent compared with primary tumors, especially in the mesenchymal subtype due to NF-1 deficiency (296, 337).

Due to their importance in promoting tumor progression and immunosuppression, therapeutic targeting of TAM and their function is being attempted. Choi et al. have summarized different molecular targets in preclinical and clinical investigations (338). These include blocking recruitment of TAM into the tumor by targeting chemokines such as CCL2 and CXCL12, which resulted in a reduction in tumor size. Further approaches target the functional characteristics of macrophages, by utilizing small-molecule inhibitors for PI3K, Ras/MAPK signaling, or the IDO pathway, leading, e.g., to reduced IL-10 secretion from M2 macrophages.

A different approach is the reversion of the M2 phenotype to the proinflammatory M1 phenotype by using, e.g., oncolytic virotherapy. Van den Bossche et al. cultured human M2 macrophages with cancer stem cells infected with Delta24-RGD virus and observed a transition towards the M1 phenotype of the cells. Patients treated with this viral particles showed an increase in M1 macrophages in their tumor tissue compared with untreated controls (339). Saha et al. demonstrated that the application of an oncolytic herpes simplex virus (oHSV) expressing IL-12 in combination with antibodies against CTLA-4 and PD-L1 shows a regression of almost all tumors in two GBM mouse models by inducing an effector T-cell influx and an increase in the M1 phenotype of macrophages (340). Thus, local immunostimulatory conditions in the tumor may alter the immunosuppressiveness of the TME. Indeed, DCV in a mouse model revealed a reduction of TAM and MDSC after treatment (341). Moreover, Dammeijer et al. reported that the kinase inhibitor PLX3397 (pexidartinib), targeting CSF-1R signaling, results in a reduction of TAM in a mouse model for malignant mesothelioma but did not influence survival. However, when combined with DCV, survival was increased, which was associated with a reduction of TAM and an increase in effector T-cell infiltration (342). These results suggest that combining DCV with depletion, blocking, or re-polarization of TAM may improve efficacy of the treatment of GBM.

Immune Checkpoint Regulation

T-cell activation by DC for antitumoral immunity requires the differentiation and expansion of antigen-experienced effector memory T cells (343), with interferon-γ (IFNγ)-producing $T_{Helper}1$ (T_H1) cells being required for efficient induction of antitumoral effector cytotoxic T cells (121). Antigen-experienced effector memory T cells are characterized by the expression of the

surface markers CD45RO and CD69 (and CD103 on tissue resident cells) in the absence of CCR7 (C-C chemokine receptor type 7) and CD62L (343–345). Effector function of these T_H1 and T_c1 cells is defined by the expression of the transcription factors (TF) T-bet and Eomes, and the effector potency depends on a delicate balance of these two TFs (346). Highly potent effector T cells present with a T-bet^{high}/Eomes^{low} profile. They produce high levels of IFNγ, perforin, and granzyme B (347).

The activity of these effector T cells needs to be tightly regulated in order to prevent excessive immune reactions and uncontrolled inflammation, which may cause destruction of healthy tissue. Therefore, in the course of activation, T cells upregulate immune checkpoint receptors on their surface, including PD-1, CTLA-4, Lag-3, and Tim-3 that upon interaction with their ligands provide a negative feedback to attenuate proliferation and function of the activated T cells, thereby preventing overreactions (348, 349).

In various cancers, including GBM, these immune checkpoint mechanisms, which normally promote self-tolerance and protect against autoimmunity, contribute to tumor immune escape (348–350). Tumor cells or components of the TME such as TAM, Treg, and MDSC express ligands of immune checkpoint receptors, which upon interaction with their receptors on tumor infiltrating T cells cause partial dysfunction of the T cells, a process also referred to as "T-cell exhaustion," because similarly dysfunctional T cells can be found in chronic infections and after repetitive T-cell stimulation (348, 349, 351). Compared with CD8$^+$ T cells generated in response to acute infections, such as an acute CMV infection, exhausted antigen-specific CD8$^+$ T cells generated in response to chronic infections or cancer are characterized by reduced proliferation rates, diminished cytotoxicity, and lower cytokine production. Additionally, they express non-transiently checkpoint receptors. T-cell dysfunction has been observed in an early stage of cancer, and it becomes more severe upon tumor progression (352, 353), protecting the tumor cells from the effector mechanisms of the T cells.

In GBM, PD-1/PD-L1 is the best characterized immune checkpoint mechanism. In the majority of tumors, cells of the TME (354) as well as tumor cells express PD-L1 (355), although expression may be restricted to a minor subpopulation of tumor cells only [0%–87%; median 2.8%; (356)]. The respective receptor, PD-1, is expressed on tumor-infiltrating CD4$^+$ and CD8$^+$ T cells (355). Expression on the tumor-infiltrating T cells is higher than on their counterparts in blood (346, 357), and they are functionally impaired (346, 358). Besides PD-1, expression of Tim-3, Lag-3, and CTLA-4 has also been observed on infiltrating T cells in GBM (359).

The dysfunctional state of the T cells may not be permanent but appears to have to be maintained by receptor–ligand interactions, in contrast to T-cell senescence, which is not reversible (353, 360). When the respective immune checkpoint receptors are blocked, e.g., by receptor or ligand-specific monoclonal antibodies, T cells can be reinvigorated: their function and proliferation are restored (359, 361). Indeed, in many tumor entities, application of monoclonal antibodies blocking immune checkpoint receptors or their ligands has resulted in a survival benefit for the patients (361).

In mouse models of GBM, blocking of CTLA-4, PD-1/PD-L1, and TIGIT has been shown to result in increased survival, frequently associated with depletion of immunosuppressive cells and an influx of effector T cells into the tumor (222, 228–231). Thus, there appears to be an intrinsic antitumoral immune response in cancer patients, which can be enhanced by interference with the immune checkpoint pathways. Although this type of immunotherapy results in durable responses in many tumors, this is only true for a fraction of patients (20%–50%, depending on the cancer type), and therapy is associated with severe immune-related adverse events (362). In contrast to many other tumors, however, in GBM, interference with immune checkpoint pathways has not been successful. No survival benefit has been reported so far in several clinical trials (363–367), except for one of three trials applying an anti-PD-1 monoclonal antibody in a neoadjuvant setting (368–370). The reason for the therapeutic failure of immune checkpoint interference in GBM is currently unknown. Whether other checkpoint regulators or possibly other immune escape mechanisms play a more prominent role in GBM than in other tumors and therefore the loss of PD-1 signaling due to blocking is irrelevant remains to be determined. However, reinvigoration of exhausted T cells may also have limitations in GBM. It depends on how terminally differentiated/exhausted T cells are. The function of exhausted T cells showing a T-bet^{low}/Eomes^{high} TF expression and a concomitant expression of multiple immune checkpoint receptors cannot be restored (346). It is further essential to distinguish between progenitor exhausted T cells, which express the TF TCF1 and the TCF1$^-$/Tim-3$^+$ terminally exhausted T cells, which co-express the TF TOX, vital for their persistence in the tumor environment with chronic antigen stimulation, with the later population not being re-invigoratable, thus not responding to checkpoint blockade (352, 353, 371). It has been suggested that immune checkpoint inhibition improves OS by overcoming the exhaustion state of tumor-infiltrating T cells resulting in an increased effector response, but functional proof about that is still missing up to date. Although several studies have described an increase in TCF1$^+$ tumor-infiltrating T cells, it is unclear whether they are re-invigorated from the exhausted cells, represent new "non-exhausted" or "non-terminally differentiated" clonotypes, or are recruited from the periphery following immune checkpoint inhibition.

Furthermore, immune checkpoint interference can only enhance but not induce antitumoral responses. The low mutational load [(94); for review, see (95)] and the low frequency [20%–30% (50, 83)] of preexisting antitumoral T-cell responses in GBM patients may therefore limit efficacy of immune checkpoint interference as well. In agreement with this, PD-1 or PD-L1 blockage combined with DCV in mouse models resulted in CD8$^+$ T cell-dependent long-term survival, which was not observed with the respective monotherapies (372, 373). Similar results have been obtained by Wang et al., who vaccinated GBM patients with personalized TAA-pulsed DC combined with low-dose cyclophosphamide, poly(IC), imiquimod, and anti-PD-1

antibody, which induced antigen-specific $CD4^+$ and $CD8^+$ T-cell responses, which were associated with a favorable outcome when compared with the respective monotherapies (86). Moreover, Jan et al. and Yao et al. reported for DCV that a lower $PD\text{-}1^+$:$CD8^+$ ratio in TIL as well as in blood lymphocytes is associated with longer survival (78, 81), and Fong et al. described an association of decreased CTLA-4 expression with survival after DCV (93). Thus, there appears to be a rationale for combining DCV and blocking of immune checkpoint regulatory pathways to increase efficacy. DCV itself might be able to trigger the expansion of the above-described $TCF1^+/TOX^-$ neoantigen-specific T cells from the periphery or even the tumor site and hence enhance tumor killing and survival.

Overall, targeting any one of the three immunosuppressive cell populations in the TME of GBM as well as the immune checkpoint regulatory pathways (**Figure 2**) appears to represent a promising approach by itself, but in particular in combination with DCV.

CONCLUSION

Even after more than 10 years of DCV in GBM and after more than 1,000 patients having been vaccinated, it is still difficult to draw conclusions as to the efficacy of DCV. However, there are promising results urging to further develop it as a therapeutic tool, which will require not only optimizing the DC vaccines in respect to target antigen selection, preparation of the cells, and integration of DCV into other treatment regimens but also dosing and scheduling of the vaccination(s). Future vaccination strategies will have also to take into account immunosuppression in GBM and the means to overcome it, which are now becoming increasingly available, to improve efficacy in GBM patients.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the article and approved the submitted version.

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Anti-Mouse CD83 Monoclonal Antibody Targeting Mature Dendritic Cells Provides Protection Against Collagen Induced Arthritis

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Antibodies targeting the activation marker CD83 can achieve immune suppression by targeting antigen-presenting mature dendritic cells (DC). This study investigated the immunosuppressive mechanisms of anti-CD83 antibody treatment in mice and tested its efficacy in a model of autoimmune rheumatoid arthritis. A rat anti-mouse CD83 IgG2a monoclonal antibody, DCR-5, was developed and functionally tested in mixed leukocyte reactions, demonstrating depletion of $CD83^+$ conventional (c)DC, induction of regulatory DC (DCreg), and suppression of allogeneic T cell proliferation. DCR-5 injection into mice caused partial splenic cDC depletion for 2–4 days (mostly $CD8^+$ and $CD83^+$ cDC affected) with a concomitant increase in DCreg and regulatory T cells (Treg). Mice with collagen induced arthritis (CIA) treated with 2 or 6 mg/kg DCR-5 at baseline and every three days thereafter until euthanasia at day 36 exhibited significantly reduced arthritic paw scores and joint pathology compared to isotype control or untreated mice. While both doses reduced anti-collagen antibodies, only 6 mg/kg achieved significance. Treatment with 10 mg/kg DCR-5 was ineffective. Immunohistological staining of spleens at the end of CIA model with CD11c, CD83, and FoxP3 showed greater DC depletion and Treg induction in 6 mg/kg compared to 10 mg/kg DCR-5 treated mice. In conclusion, DCR-5 conferred protection from arthritis by targeting CD83, resulting in selective depletion of mature cDC and subsequent increases in DCreg and Treg. This highlights the potential for anti-CD83 antibodies as a targeted therapy for autoimmune diseases.

Keywords: CD83, dendritic cells, regulatory T cells, antigen presentation, collagen induced arthritis (CIA), mouse, monoclonal antibody

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Abbreviations: ADA, anti-drug antibodies; ADCC, antibody dependent cell cytotoxicity; AF, Alexa Fluor; APC, antigen presenting cell; cDC, conventional dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; CIA, collagen induced arthritis; CTV, cell trace violet; DC, Dendritic Cells; DCreg, regulatory dendritic cell; FL-DC, Flt-3L cultured bone marrow DC; i.p., intra-peritoneal; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; pDC, plasmacytoid dendritic cell; RA, rheumatoid arthritis; Tcon, conventional T cell; Treg, regulatory T cell.

INTRODUCTION

Dendritic cells (DC) are a rare heterogeneous immune population that can be broadly separated into conventional (c) and plasmacytoid (p)DC subsets. These cells constantly survey their tissue environments for antigens. Antigens are internalized, processed, and transported by DC to lymphoid organs where they are presented as peptides *via* major histocompatibility complex (MHC) molecules to T cells. On encountering antigen accompanied with inflammatory cytokines or danger signals triggering pattern recognition receptors, DC mature (1). This results in cytokine and chemokine secretion and upregulation of surface coreceptors required to activate specific T cell responses. In the absence of danger signals or inflammatory cytokines, antigen presentation by immature DC directs T cell tolerance through mechanisms such as deletion, anergy or induction of regulatory T cells (Treg) (2). Alternatively, signaling through anti-inflammatory cytokines such as IL-10, homeostatic signals through vitamin D or reverse signaling through costimulatory receptors CD80/86 guide DC to mature into a regulatory state (known as DCreg) that actively induce T cell tolerance (3–5). Inappropriate maturation of DC presenting self-antigens or ineffective induction of DCreg can lead to activation of autoreactive T cells causing autoimmune diseases (6, 7).

Rheumatoid arthritis (RA) is a debilitating systemic autoimmune disease causing inflammation, pain, and ultimately loss of joint function that affects approximately 1% of adults worldwide (8). While targeted therapies with limited immune suppression have become common and effective, therapies that target novel pathways involved in the initiation of the immune response (e.g., antigen presentation) are desirable to arrest disease at an earlier stage. Collagen induced arthritis (CIA) is a mouse model of this disease driven by mature DC in lymphoid organs and synovial fluid initiating self-reactive T cell and B cell responses through antigen presentation and coincidental inflammatory cytokine and chemokine secretion (9, 10). In arthritis mouse models, adoptive transfer of DC prevented from maturing by genetic or chemical means suppressed arthritogenic T and B cell activation by inducing Treg (11–13), a mechanism which has been translated to human clinical trials (14, 15). Targeting mature DC *in situ* with antibodies is a potential strategy for treating RA.

CD83 is an Ig-superfamily molecule most highly expressed on the surface of mature DC, but also present transiently at lower levels on other activated antigen presenting cells (APC) such as B cells and subsets of activated T cells (16, 17). CD83 contributes to APC maturation by binding and sequestering the ubiquitinase ligase MARCH1, inhibiting the ability of the enzyme to ubiquitinate and degrade surface MHC class II and CD86 molecules (18). CD83 is also released in a soluble form with demonstrated immunoregulatory properties (17). In order to therapeutically target mature DC in inflammatory diseases, our group developed antibodies to the human CD83 molecule, first as a polyclonal reagent to demonstrate proof of principle (19, 20), and then as a therapeutic affinity matured human IgG1, Fc-competent monoclonal antibody (mAb), 3C12C (21). Both reagents facilitated antibody-dependent cell cytotoxicity (ADCC) killing of $CD83^+$ targets including human DC populations *in vitro*, preventing alloreactive T cell presentation and proliferation in mixed leukocyte reaction (MLR) assays. The antibodies also target DC populations in human peripheral blood mononuclear cell (PBMC) xenografted SCID mice, effectively suppressing xenogeneic T cell responses, thereby preventing graft-versus-host disease. Importantly, anti-CD83 antibodies preserved xenogeneic anti-viral and anti-tumor T cell responses and did not target xenogeneic Treg or immature DC that enhance self-tolerance (20, 21). 3C12C has shown similar functional characteristics and a good safety profile in a non-human primate model (22). Another group showed that a mouse anti-human CD83 mAb could deplete DC and restrain self-reactive T cell generation in a xenograft temporal-artery plus PBMC SCID mouse model of autoimmune vasculitis indicative of potential utility for anti-CD83 treatment in autoimmune diseases (23).

To gain a better understanding of the immunoregulatory mechanisms of anti-CD83 antibodies, we generated a rat anti-mouse CD83 mAb, DCR-5, that mimics the properties of our human anti-human CD83 therapeutic 3C12C. Using *in vitro* and *in vivo* assays, we showed that the DCR-5 antibody has the potential to deplete mature $CD83^+$ cDC and induce DCreg, leading to reduced T cell activation and greater Treg induction. This immune suppressive function of DCR-5 was effective in decreasing the severity of arthritis in the CIA mouse model of disease.

METHODS

Mice

For the CIA model, DBA/1 male mice were ordered from Envigo (Indianapolis, IN) and housed at the Bolder BioPath (Boulder, CO) facility under specific pathogen free conditions. All other mice were housed at the ANZAC Research Institute (Sydney, Australia) under specific pathogen free conditions. C57BL/6JAusb (B6), BALB/cJAusb (BALB/c), and B6.SJL-$Ptprc^aPepc^b$/BoyJAusb (B6.$Ptprc^a$) mice (24) were purchased from the Animal BioResources (Moss Vale, Australia) and DBA/1J mice were purchased from the Walter and Eliza Hall Institute (Parkville, Australia). B6.129S4-$Cd83^{tm1Tft}$/J (CD83KO) mice (25) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Production of Anti-CD83 mAb

Rat hybridomas were generated by the Monash Antibody Technologies Facility (Clayton, Australia). Four Wistar rats were immunized intra-peritoneally (i.p.) three times at 2-weekly intervals with 16 μg recombinant His-tagged extracellular domain (Met 1-Arg 133) of mouse CD83 (Sino Biologicals, Beijing, China) and Sigma Adjuvant System plus methylated CpG (Sigma-Aldrich, St. Louis, MO). Splenocytes from rats were fused to the mouse myeloma cell line SP2/0-Ag14 using a conventional polyethylene glycol fusion protocol. IgG-producing hybridomas from the first two rats were selected for binding to recombinant mouse CD83-Fc (Sino Biological) by ELISA. Two additional rounds of selection were performed on lipopolysaccharide (LPS; *In vivo*gen, San Diego CA) stimulated (1 μg/ml, 4 h) A20 cell line (ATCC; Manassas, VA) by flow cytometry culminating in the DCR-3 clone. IgG hybridomas

from the other two rats were selected by flow cytometry comparing binding to CRISPR/Cas9 CD83 deleted and parent WT mutuDC 1940 strain [kindly provided by Justine Mintern and Hans Acha-Orbea (26)] stimulated for 18 h with 1 μM CpG-ODN 2395 (*In vivo*gen), culminating in the selection of the DCR-5 clone. DCR-3 and DCR-5 hybridomas were adapted to serum free media (Hybridoma-SFM; ThermoFisher, Waltham, MA) before being grown in CELLine Bioreactor flasks (Wheaton, Staffordshire, UK) to produce bulk quantities of antibodies that were purified by Protein G affinity chromatography (GE Healthcare, Chicago IL) on an NGC Affinity Chromatography System (Bio-Rad, Hercules CA). Antibodies were passed through 0.22 μm filters (Interpath, Pendleton, OR) and isotypes determined by rat mAb isotyping test kit (AbD Serotec, Kidlington, UK). The average purity of antibody bands in DCR-5 preparations were determined on average to be 95.1% over multiple reads using a Bioanalyzer Agilent Protein 230 Chip (Agilent Technologies).

Flow Cytometry

CD83 binding of purified DCR-3, DCR-5 or Michel-19 (BD Biosciences, San Jose, CA) antibodies were examined at 20 μg/ml and detected with a goat anti-rat IgG-AlexaFluor (AF)-488 secondary antibody (ThermoFisher). Equal amounts of purified rat IgG1/κ (R3-34), rat IgG2a/κ (R35-95) or rat IgG2b/κ (A95-1) isotype controls (BD Biosciences) were used for comparison. Rat serum (10%) and Fc Block (2.4G2; BD Biosciences) were used to respectively block anti-rat antibodies and Fc receptors before staining with combinations of the fluorochrome-labeled anti-mouse antibodies: CD4-Brilliant Violet (BV)650 (RM4-5), CD8a-peridinin-chlorophyll-protein (PerCP)-Cy5.5 (53-6.7), CD11b-V500 (M1/70), CD11c-phycoerythrin (PE)-Cy7 or allophycocyanin-Cy7 (N418), CD25-PE (PC61.5), CD40-PerCP-Cy5.5 (3/23), CD45.1-BV650 (A20), CD45.2-PerCP-Cy5.5 (104), CD45R/B220 (RA3-6B2)-allophycocyanin, CD80-PE-CF594 (16-10A), CD86-PE (GL-1), F4/80-fluorescein isothiocyanate (FITC) (BM8); H-2K^b-FITC (AF6-88.5), IA/IE-PE or AF700 (M5/114-15.2), Ly-6C-PerCP-Cy5.5 (HK1.4), PDCA-1- allophycocyanin (JF05-1C2.4.1), PD-L1-BV711 (Ty75), PD-L2-allophycocyanin (10F.9G2) from BD Biosciences, ThermoFisher, BioLegend (San Diego, CA) or Miltenyi Biotec (Bergisch Gladbach, Germany). The Michel-19 anti-CD83 clone on FITC (Biolegend) or BV711 (BD Biosciences) was used to assess CD83 expression in DCR-5/isotype control treated samples. Lineage (Lin) specific biotinylated antibodies CD3e (145-2C11), CD19 (1D3), Ly6G (1A8) and NK1.1 (PK136) were detected using streptavidin-BV421 (BioLegend or BD Biosciences). Cell viability was assessed with 3 μM DAPI or Zombie Aqua (ThermoFisher) staining. After surface staining, intracellular FoxP3-allophycocyanin (FJK-16s), IDO1-AF647 (2E2/IDO1) or IL-10-BV650 (JES5-16E3) staining was performed using the Foxp3/Transcription Factor Staining Buffer Set Kit (ThermoFisher). Cells were stimulated with 50 ng/ml phorbol myristate acetate and 1 μg/ml ionomycin (Sigma Aldrich) with BD Golgi-Plug (BD Biosciences) for 4 h prior to staining. T cells were labeled with 0.5 μM Cell Trace Violet (CTV) or carboxyfluorescein succinimidyl ester (CFSE; ThermoFisher) to assess proliferation. Data was collected on a BD LSR Fortessa flow cytometer and analyzed on FlowJo 10 software (BD Biosciences).

In Vitro Assays

All assays were performed in RPMI 1640 media supplemented with 10% fetal calf serum, 2 mM GlutaMAX, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES and 50 mM 2-mercaptoethanol (all ThermoFisher). DC and B cell activation was achieved by culturing red blood cell depleted splenocytes (2×10^6 cells/ml) in 1 μg/ml LPS or 5 μM CpG-ODN 2395 for 18 h. T cells were activated by culturing 2×10^5 T cells with 1×10^5 anti-CD3/28 Dynabeads (ThermoFisher) for 18 h. For DC depletion, the indicated concentrations of antibodies were added to an MLR in which 5×10^5 B6 and 5×10^5 BALB/c or DBA/1 splenocytes (i.e., 1:1 ratio) in the presence of 100 U/ml human IL-2 were cocultured in 96-well plates for 18 h. The T cell proliferation MLR assay was performed as above with CTV labeled B6.*Ptprc*^{a} splenocytes, with cells cultured for 84 h. Purified low-endotoxin azide-free rat IgG2a/κ (R35-95) or rat IgG2b/κ (A95-1) mAb (BD Biosciences) were used as isotype controls. The mouse T helper cell cytometric bead array (BD Biosciences) was used to measure cytokines in MLR supernatants using manufacturer's instructions. For Treg suppression assay, CD4$^+$ CD25$^+$ T cells were purified by MACS from spleens of B6 mice treated i.p. with 150 μg DCR-5 or anti-trinitrophenol rat IgG2a/κ mAb (InVivoPlus 2A3, BioXcell, Lebanon NH) isotype control following the CD4$^+$CD25$^+$ regulatory T cell isolation kit instructions (Miltenyi Biotec). FoxP3 was confirmed on 94–96% of isolated cells from both strains. The CD4$^+$CD25$^-$ conventional T cell (Tcon) fraction was purified from spleens of untreated B6.*Ptprc*^{a} mice using the same kit and labelled with 0.5 μM CFSE. Labeled Tcon (2.5×10^4) were cultured with DCR-5 and isotype control treated Tregs at the indicated ratios with 12.5×10^4 mouse T activator CD3/CD28 Dynabeads (ThermoFisher). CD45.1$^+$CD4$^+$ Tcon proliferation was assessed by CFSE dilution after 3 days using flow cytometry. Flt-3L cultured bone marrow DC (FL-DC) were generated by culturing B6.*Ptprc*^{a} bone marrow with complete RPMI containing 12.5% B16 cell line supernatant as described (27). DC (2.5×10^6 cell/ml) were washed and re-cultured with 20 μg/ml of DCR-5 or rat IgG2a/κ mAb (InVivoPlus 2A3, BioXcell) isotype control in complete RPMI overnight to assess DC surface marker expression. To assess Treg induction, antibody treated DC were washed and then re-cultured for 84 h with 1×10^5 BALB/c T cells (0.5 μM CFSE labeled for proliferation or non-labeled for intracellular staining) at a 1:8 ratio.

In Vivo Assays

B6 or DBA/1 mice were injected i.p. with 200 μl PBS containing the indicated amount of DCR-5, rat IgG2a (InVivoPlus 2A3, BioXcell) isotype control or untreated. Mice were euthanized for analysis when indicated.

Immunoprecipitation and Immunoblot

B6 FL-DC stimulated with 1 μg/ml LPS overnight were washed and incubated with a 1 mg/ml sulfo-N-hydroxysulfosuccinimide (NHS) biotin in PBS (pH 8.0) solution. The biotinylation reaction was inactivated by washing the cells with 100 mM glycine in PBS before

being lysed in mammalian protein extraction reagent (ThermoFisher). Protein A conjugated Dynabeads (ThermoFisher) coated with 10 μg rat IgG2a isotype control (2A3, BioXcell) were incubated with the lysate to clear it of non-specific binding proteins. The beads were removed using a magnetic column. Dynabeads coated with 10 μg purified DCR-5 or Michel-19 (BD Biosciences) antibody were then used to immunoprecipitate CD83 from lysate. Target protein was eluted reduced and denatured prior to being run on a 4–12% Bis-Tris Plus gel (ThermoFisher) and transferred to nitrocellulose membrane using the iBlot system (ThermoFisher). Membranes were blocked with 5% skim milk powder in TBST and incubated with Streptavidin-HRP (BioLegend) to detect biotinylated cell surface derived proteins. Alternatively, recombinant His-Tag mouse CD83 or CD302 (Sino Biological) were immunoprecipitated with DCR-5 or Michel-19 coated Dynabeads and immunoblotted with anti-His Tag-HRP (BioLegend). Signal was detected with an ECL reagent (Clarity ECL kit; Bio-Rad) and visualized using the Chemidoc image system (Bio-Rad). SeeBlue protein standards (ThermoFisher) were used for size comparison.

CIA Model

The CIA model was performed by Bolder BioPath. DBA/1 male mice immunised intradermally into the tail base with 200 μg bovine type II collagen (Bolder BioPath) and 250 μg complete Freunds' adjuvant (MP Biomedical, Irvine CA). I.p. treatment with the indicated concentrations of DCR-5 or isotype control was initiated on d0 and continued every 3 days. Control groups included naïve non-immunized mice or immunized mice that were either untreated or treated with dexamethasone (0.2 mg/kg; VetOne, Boise ID) subcutaneously every second day. Body weights (d0-36) and mean clinical scoring of four paws (d21-36) were recorded: 0 = Normal; 1 = 1 hind or fore paw joint affected or minimal diffuse erythema and swelling; 2 = 2 hind or fore paw joints affected or mild diffuse erythema and swelling; 3 = 3 hind or fore paw joints affected or moderate diffuse erythema and swelling; 4 = marked diffuse erythema and swelling, or 4 digit joints affected; 5 = severe diffuse erythema and severe swelling of the entire paw, unable to flex digits. On d36, animals were anesthetized and exsanguinated followed by cervical dislocation to collect spleen, paw and knee tissues for histological analysis. Timed euthanasia was performed on an additional untreated group, with two mice euthanized pre-immunization and remaining mice euthanized at days 1, 2, 9, 22, and 36 post-immunization.

Histology

Histopathologic evaluation of knee and paw tissue from CIA studies were performed by HistoTox Labs (Boulder, CO). Tissues were fixed 1–2 days in 10% formalin and decalcified 4–5 days in 5% formic acid before being embedded in paraffin. Sections were stained with toluidine blue and images were acquired using a Axioscan Z1 slide scanner and analysed using Zen software (Zeiss, Oberkochen, Germany). Inflammation, pannus formation, cartilage damage, bone resorption, and periosteal new bone formation were scored (0–5) in a blinded fashion as described (28). OCT snap frozen spleen sections were dried overnight, fixed with ice-cold acetone, and blocked with 1% BSA in PBS before staining. For immunofluorescence, spleen sections were incubated with biotinylated anti-CD83 (Michel-19; BioLegend) and subsequently with streptavidin-AF488 (ThermoFisher), anti-CD11c-AF594 (N418; BioLegend) and anti-CD45R-AF647 (RA3-6B2; BioLegend), washing with 0.5% BSA in PBS in between. For immunohistochemistry, endogenous peroxidase in spleen sections was quenched with 0.3% H_2O_2/PBS. The sections were blocked with an Avidin/Biotin blocking kit as per manufacturer's instructions (Vector Labs, Burlingame CA) and then individually stained with biotinylated CD11c (N418; BioLegend), FoxP3 (FJK-16s; ThermoFisher) or PBS alone, detected using a Streptavidin-HRP (BioLegend) secondary and DAB Peroxidase Substrate (Vector Lab, Burlingame, CA). Each section was finally counterstained with 10% Harris hematoxylin solution (Australian Biostain, Traralgon, Australia). Tissue sections were imaged on an EVOS-FL II Cell Imaging System (ThermoFisher) and then processed and analyzed with Image J software (NIH).

ELISA

For anti-CD83 ELISAs, plates were coated overnight with 1 μg/ml mouse CD83-Fc (Sino Biologicals) and blocked with 5% BSA. Plates were overlayed with rat anti-mouse CD83 antibody, 1:100–1:1,000 diluted serum or hybridoma supernatant (50 μl) and then an HRP conjugated goat anti-rat IgG Fc-specific antibody (Sigma-Aldrich). The HRP substrate SIGMAFAST OPD solution (Sigma-Aldrich) was used to detect binding of antibodies by reading absorbance at 450 nm on a Victor3 Multilabel Plate Reader (Perkin Elmer). Wells were washed with 0.05% Tween 20 in PBS between steps. To detect anti-DCR-5 antibodies, ELISA plates were coated with 10 μg/ml DCR-5 and blocked with 5% BSA. Plates were overlayed with serum samples diluted 1:10,000 and 1:100,000 in PBS, without incubation (no spike) or after incubation for 30mins with 10μg/ml DCR-5 (spike). Bound mouse antibodies were detected with HRP conjugated pre-adsorbed goat anti-mouse IgG (ab97040, Abcam, Cambridge, MA), measured as above. Anti-collagen antibodies produced in the CIA model were measured in 1:500 and 1:40,000 diluted serums using the mouse anti-type I/II collagen IgG assay kit with TMB (Chondrex, Redmond WA) under manufacturers' instructions. The mouse IL-10 ELISA (ThermoFisher) performed on FL-DC supernatants was conducted using the manufacturer's instructions.

Statistics

Statistical tests include t-tests or one and two-way ANOVA for comparison of two or multiple groups, respectively, as indicated in figures and performed in GraphPad Prism (San Diego, CA). Correlation was assessed by calculating Pearson's correlation coefficient. Error bars mark standard error of the mean.

RESULTS

Development of Novel Rat Anti-Mouse CD83 mAbs

Two hybridoma clones producing novel rat mAbs to mouse CD83 were selected from independent fusions and named DCR-3

(IgG2b/κ isotype) and DCR-5 (IgG2a/κ). Both bound similarly to mouse CD83-Fc coated ELISA plates compared to the commercial rat anti-mouse CD83 (IgG1/κ isotype) clone, Michel-19 (**Figure 1A**). Flow cytometry showed significant binding of Michel-19, DCR-3 and DCR-5 to native CD83 on the surface of splenic conventional (c)DC in WT but not CD83KO B6 splenocytes activated *in vitro* with LPS overnight (**Figure 1B**). Reduced binding of all three antibodies was seen on LPS-activated splenic B cells compared to splenic DC from WT mice. Comparison of binding to different APC subsets activated using LPS or CpG-ODN 2395 showed highest binding of Michel-19, DCR-5 and DCR-3 to activated $CD8^+$ cDC (DC1) followed by $CD8^-$ (CD11b+) cDC (DC2) (**Supplementary Figures 1A, B**). Lower levels of binding were found on LPS or CpG-ODN 2395 stimulated macrophages, monocytes, B cells, and plasmacytoid (p)DC. Minimal CD83 levels were detected on unactivated subsets. Similar binding profiles by these mAbs were seen on LPS activated cDC and B cells from DBA/1 (**Supplementary Figure 1C**) and BALB/c mice (not shown). DCR-3/5 did not bind to conventional $CD4^+$ or $CD8^+$ T cell populations that were unactivated or after 18 h of CD3/CD28 stimulation, while Michel-19 showed low level binding to activated $CD25^+CD8^+$ T cells (**Figure 1C**). Michel-19 also exhibited binding to the surface of ~40% of $CD4^+CD25^+FoxP3^+$ Treg after CD3/28 stimulation, consistent with previous mouse studies (16, 29), but DCR-3 and DCR-5 displayed lower binding to this population. Cross-blocking studies on the LPS activated A20 B cell line showed that Michel-19 and DCR-3 inhibited each other's binding to CD83 indicative of proximal epitopes, whereas DCR-5 minimally blocked DCR-3 or Michel-19 binding suggesting that it bound a distinct epitope (**Figure 1D**). DCR-5 and Michel-19 both immunoprecipitated recombinant His-tagged mouse CD83 protein, which was detected in an immunoblot as a ~35 kDa protein (**Supplementary Figure 1D**). Various CD83 protein bands (33, 42, 50, 57, 68, 77, and 105 kDa) were immunoprecipitated from the biotinylated cell surface of LPS stimulated mouse FL-DC (**Supplementary Figure 1E**).

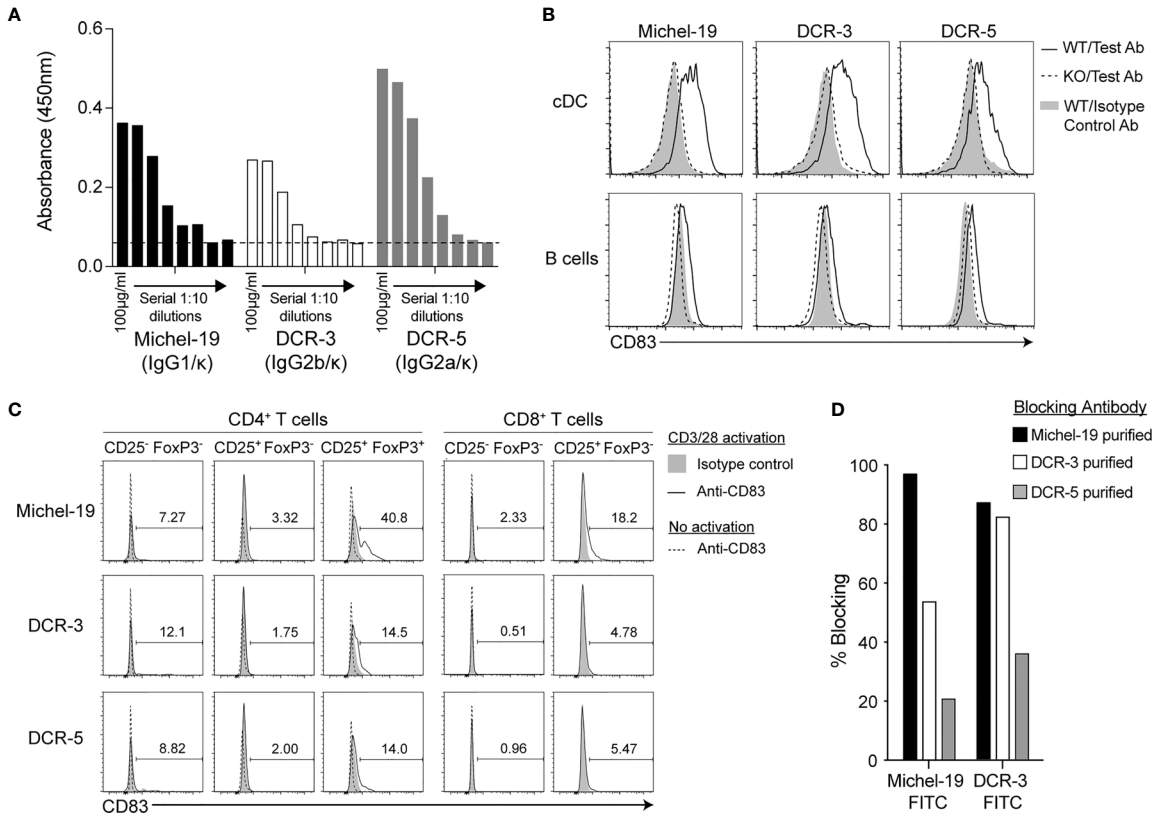

FIGURE 1 | Binding properties of rat anti-mouse CD83 antibodies DCR-5 and DCR-3. **(A)** Comparison of binding of DCR-5, DCR-3 and Michel-19 anti-CD83 antibody clones (1:10 serial dilutions starting at 100 µg/ml) to plates coated with recombinant mouse CD83-His by ELISA. Dashed line marks the absorbance in the no antibody control. Binding of 20 µg/ml Michel-19, DCR-3 or DCR-5 antibody (or respective isotype controls) was compared on the surface of **(B)** gated cDC and B cells (see **Supplementary Figure 1A** for gating strategy) in WT and CD83KO splenocytes cultured overnight with 1 µg/ml LPS or; **(C)** gated $FoxP3^-CD25^-$ (naïve) and $CD25^+$ (activated) $CD8^+$ T cell cells and $FoxP3^-CD25^-$ (naïve), $FoxP3^-CD25^+$ (activated) and $FoxP3^+CD25^+$ (Treg) $CD4^+$ T cell subsets cultured with or without overnight stimulation with CD3/CD28 microbeads. Primary antibodies were detected using an anti-rat IgG (Fc-specific)-AF488 antibody *via* flow cytometry. Percentage of $CD83^+$ cells in CD3/28 stimulated cultures shown. No $CD8^+CD25^+$ T cells detected in unstimulated cultures. **(D)** Binding of sub-saturating concentrations of FITC conjugated Michel-19 or DCR-3 to LPS stimulated A20 cells by flow cytometry was compared with and without initial blockade with saturating concentrations of purified Michel-19, DCR3 or DCR-5. Degree of blocking was calculated as percentage reduction in MFI with 0% indicating no blocking (MFI equivalent to anti-CD83 FITC antibody alone) and 100% indicating full blocking (MFI equivalent to binding of isotype-FITC antibody).

Corresponding bands were isolated with Michel-19, with less efficiency. Three of these bands (50, 57, and 77kDa) were immunoprecipitated from the cell surface of LPS stimulated A20 cells using DCR-5 and Michel-19 (data not shown). The assortment of CD83 bands detected by DCR-5 and Michel-19 mirrored the multiple CD83 isoforms detected by anti-human CD83 antibodies HB15a, HB15e, and 3C12C in immunoblots (16) and likely represent variations in glycosylation or splice variants of CD83.

DCR-5 Depletes CD83$^+$ cDC, Induces DCreg and Inhibits CD4$^+$ T Cell Proliferation in MLR Assays

To determine the functional activity of DCR-3 and DCR-5, their effects on activated APC and allogeneic T cell proliferation were examined when added to MLR between B6 and BALB/c splenocytes. After 18 h of culture, surface CD83 expression was observed highest on B6 and BALB/c cDC, at lower levels on B cells, but not on T cells within the MLR (**Supplementary Figure 2A**). The addition of 5–40 μg/ml DCR-5 antibody to cultures produced a significant decrease in total, B6 and BALB/c cDC, with the highest concentration causing 40–45% reduction compared to isotype control or untreated cells (**Figures 2A, B**). In contrast, B cells in the MLR were minimally affected by DCR-5, with only the highest dose causing a decrease of 12%. When DCR-5 and DCR-3 were compared side by side at 10 μg/ml in MLR experiments, only the former depleted cDC (**Figure 2C**), with neither affecting B cells (not shown). The DC remaining in MLR following overnight DCR-5 treatment displayed low to no surface CD83 compared to DC in isotype control treated wells. In addition, DCR-5 treatment was associated with a small reduction in surface MHC class II together with increased CD80 and CD86 costimulatory molecules in DC compared to controls, and notably augmented expression of DCreg associated surface markers CD25 and PD-L2, and the intracellular marker IDO1 (**Figure 2D**). Similar depletion of cDC (and not B cells) and induction of CD80^{hi}/86^{hi} DC was observed when using DCR-5 in a B6 × DBA/1 strain MLR (**Supplementary Figure 2B**).

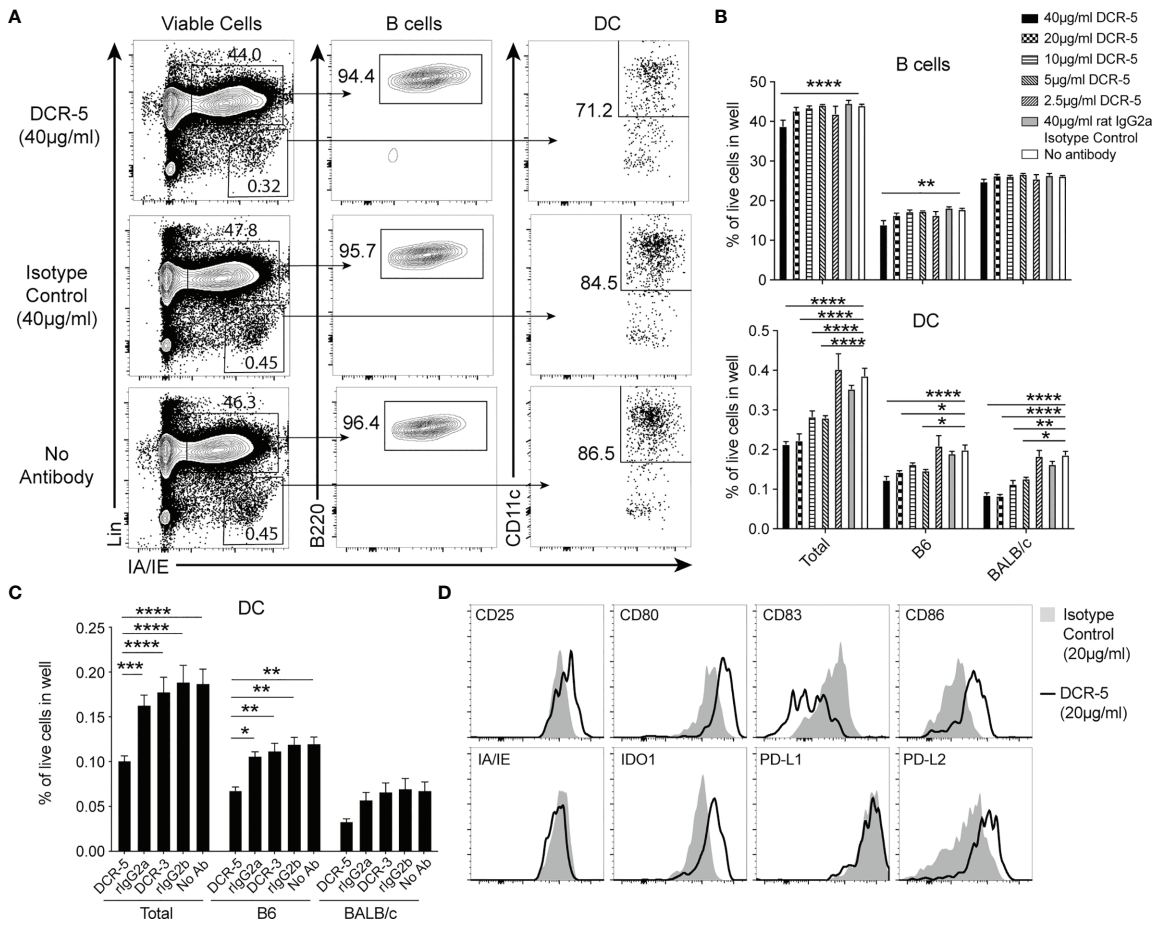

FIGURE 2 | DCR-5 depletes and alters DC phenotype *in vitro*. **(A–D)** B6 and BALB/c splenocytes were cocultured overnight in MLR at a 1:1 ratio with the indicated concentrations of DCR-5 or isotype control. Levels of DC and B cells per well were determined by flow cytometry. **(A)** Representative plots showing total DC and B cells in specified groups and **(B)** mean levels of total, B6 (H-2Kd$^-$) and BALB/c (H-2Kd$^+$) derived cells from triplicate wells. Statistical comparisons to no antibody group by 2-way ANOVA. **(C)** Mean percentage of DC in 12 wells from 3 experiments treated with no antibody or 10 μg/ml DCR-5, DCR-3 or isotype controls. Statistical comparisons by 2-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. **(D)** One of three representative plots of designated surface and intracellular DCreg markers expressed by total DC after overnight MLR culture with 20 μg/ml DCR-5 or isotype control.

DCR-3 and DCR-5 were evaluated for their effect on allogeneic T cell proliferation after 84 h in the B6 × BALB/c MLR. Adding 20 μg/ml DCR-5 reduced B6 $CD4^+$ T cells undergoing proliferation compared to the isotype control (**Figures 3A, B**) by an average of 36 ± 5.1% over five experiments (**Figure 3C**). DCR-3 failed to reduce B6 $CD4^+$ T cell proliferation and neither DCR-5 nor DCR-3 significantly affected B6 $CD8^+$ T cell proliferation in the MLR (**Figures 3A–C**). Therefore, targeting CD83 expressing cDC in MLR cultures reduced their ability to drive $CD4^+$ T cell proliferation.

We examined $CD4^+$ T cell associated cytokines in the supernatants of B6 × BALB/c T cell MLR treated with 2.5, 10, and 40 μg/ml DCR-5. These doses were confirmed to significantly decrease $CD4^+$ T cell proliferation in a dose dependent fashion compared to isotype and no antibody controls in the same assays (**Figure 3D**). The cytokines showing largest fold increase in DCR-5 treated supernatant were IL-10, IL-6, and IL-17A cytokines, with only IL-10 and IL-6 exhibiting a dose dependent response (**Figure 3E**). IFN-γ displayed a non-significant increasing trend with increasing doses of DCR-5 while TNF was only modestly induced by 2.5 and 10 μg/ml of DCR-5. IL-2 and IL-4 cytokines were both significantly inhibited by addition of DCR-5. The increase in immunoregulatory IL-10 and inhibition of mitogenic IL-2 are two possible factors that contribute to the decrease in $CD4^+$ T cell proliferation in DCR-5 treated MLR. Intracellular staining of cells 48 h into MLR showed that the source of increased IL-10 production from DCR-5 treatment came primarily from Tcon and DC, but not Treg (**Figure 3F**).

In Vivo DCR-5 Administration Reduces $CD83^+$ DC and Induces DCreg and Treg in Mice

The *in vitro* effects of DCR-5 in the MLR assays led us to examine DC targeting *in vivo*. Eight-week-old female B6 mice were injected i.p. with varying doses of DCR-5 ranging from 5 to 500 μg. Analysis of splenocytes by flow cytometry 48 h later revealed no significant effects on pDC or B cells with any dose of DCR-5 compared to isotype control or untreated animals (**Figure 4A** and **Supplementary Figures 3A, B**). However, a small decrease in frequency, but not total numbers, of cDC was seen in mice

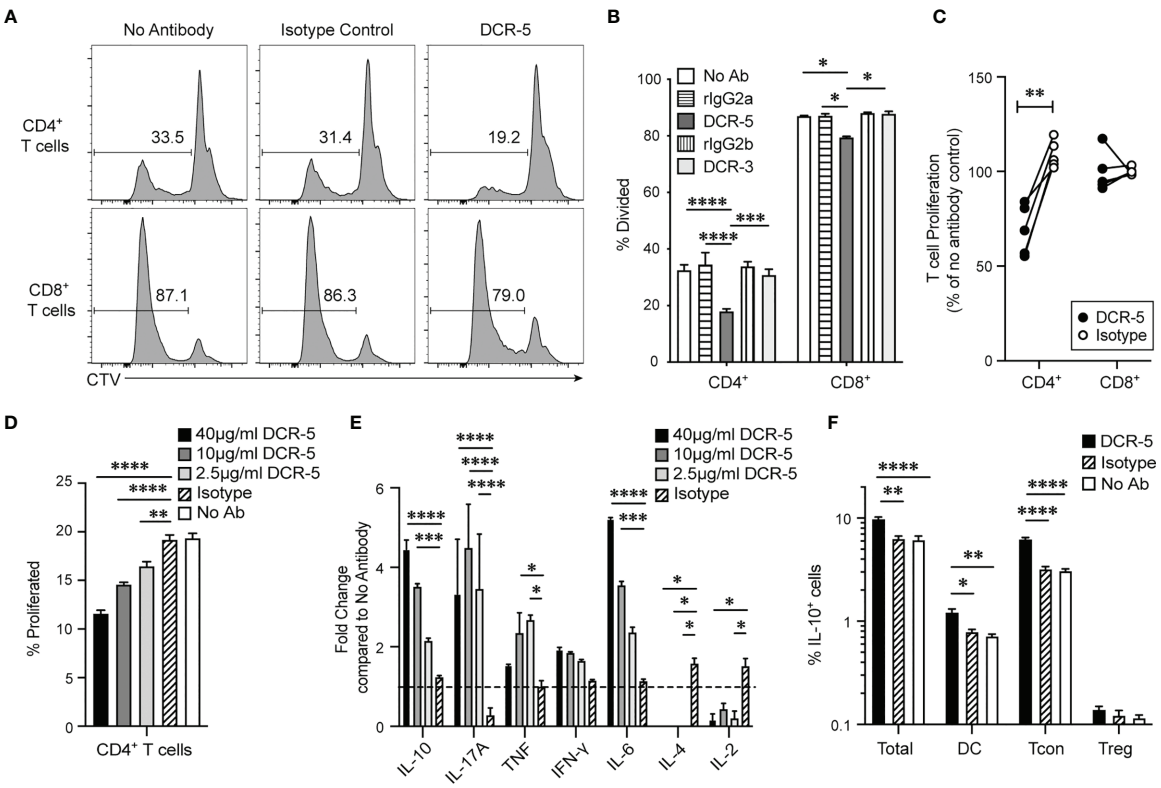

FIGURE 3 | DCR-5 prevents T cell proliferation *in vitro*. BALB/c and CTV-labeled B6 splenocytes were cocultured for 84 h in MLR at a 1:1 ratio with 20 μg/ml DCR-5, DCR-3, isotype controls or no antibody. Proliferation of gated $CD3^+CD4^+$ or $CD8^+$ B6 T cells was determined by CTV dilution. **(A)** Representative histograms showing % divided $CD4^+$ and $CD8^+$ T cells in indicated groups. **(B)** Mean levels of division in triplicate wells. Statistical comparison was determined using 2-way ANOVA. **(C)** Summary of five MLR experiments where T cell proliferation is represented as % of no antibody control. Statistical comparison was determined using a paired T-test. **(D)** $CD4^+$ T cell proliferation and **(E)** cytokines in supernatant of B6 × BALB/c MLR treated with the indicated DCR-5 concentrations or 40 μg/ml isotype control after 84 h (n = 3). Cytokine data is shown as fold change compared to mean of no antibody control (dashed line). **(F)** Percentage of total cells, $CD3^+CD4^+FoxP3^-$ (Tcon) $CD3^+CD4^+FoxP3^+$ (Treg) T cells and $CD11c^+$ $IAIE^+$ (DC) that stained IL-10^+ by intracellular flow cytometry staining after 48 h culture of B6 × BALB/c MLR with 20 μg/ml DCR-5, isotype control or no antibody. Statistical comparison to isotype control was assessed by one-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

treated with ≥ 50 μg DCR-5 (**Figures 4A–D**). A more prominent decrease was seen in the frequency (71–73% decrease) and number (49–63% decrease) of $CD83^+$ $MHCII^{hi}$ cDC in spleens (**Figures 4A–D**). It was also evident that $CD8^+$ cDC had comparatively higher depletion following DCR-5 injection than $CD8^-$ cDC. DCR-5 treated mice showed an increase in $CD80^{-/lo}$/$CD86^{-/lo}$ cDC and overall decreases in $CD80^+$ cDC ± CD86 compared to isotype control and untreated mice (**Figure 4A** and **Supplementary Figure 3C**). Interestingly, like the MLR, DCR-5 treatment induced a population of $CD80^{hi}/86^{hi}$ cDC (see oval in **Figure 4A**) not present in isotype or untreated controls. This population expressed low CD11c and were $CD83^{dim}$ compared to the remainder of cDC in DCR-5 and those in isotype treated mice (**Figure 5**). An analysis of additional markers showed that the induced $CD80^{hi}/86^{hi}$ subset did not express markers of pDC (PDCA-1, CD45R), macrophages (F4-80), monocytes (Ly6C) or innate lymphoid cells (CD117, Sca-1, CD90; not shown), but most increased CD25, PD-L1 and PD-L2 expression associated with DCreg differentiation (30). IDO1 expression was similarly expressed between $CD80^{hi}/86^{hi}$ cDC and those expressing lower CD80/86 levels in DCR-5 and isotype control treated mice. DCR-5 injected into DBA/1 mice showed a similar capacity to deplete $CD83^+$ cDC and induce a $CD80^{hi}/86^{hi}$ cDC population (**Supplementary Figure 3D**).

We next determined whether targeting $CD83^+$ cDC with DCR-5 would alter the balance of regulatory to conventional T cells in mice. No differences were seen in frequency (**Figure 6A**) or total numbers (**Figures 6B, C**) of splenic $CD4^+$ or $CD8^+$ T cells in mice treated with 50 to 500 μg of DCR-5 compared to control mice. However, DCR-5 treated mice exhibited between a 1.5- and 1.9-fold increase in splenic $CD4^+CD25^+FoxP3^+$ Treg numbers (**Figure 6D**) and 1.5–1.7-fold increase as a proportion of $CD4^+$ T cells compared to control groups (**Figures 6E, F**). No CD83 expression was detected on Treg of DCR-5, isotype treated or untreated mice (**Figure 6A**), suggesting that the DCR-5 was not expanding Treg through direct binding to this population. This was consistent with *in vitro* experiments demonstrating minimal binding of DCR-5 to Treg (**Figure 1C**). While present

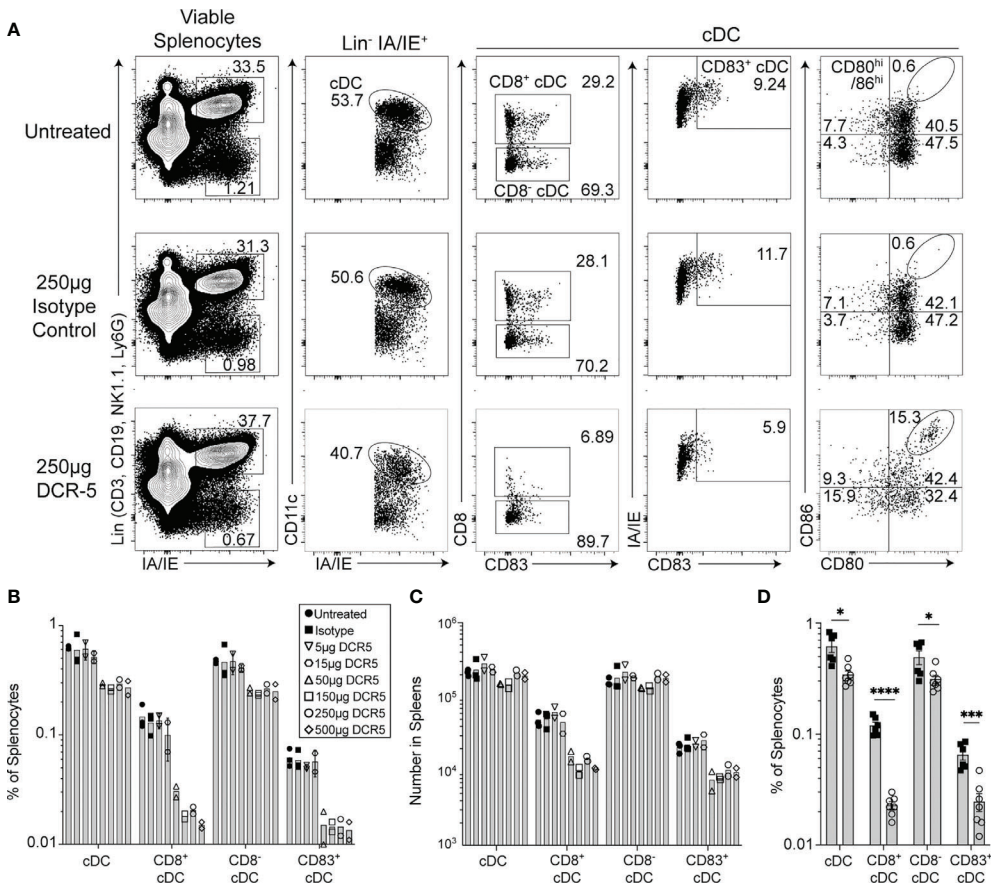

FIGURE 4 | *In vivo* treatment of mice with DCR-5 results in depletion of DC. B6 mice treated with a single i.p. injection of increasing doses of DCR-5 mAb or 250 μg rat IgG2a isotype control were analyzed 48 h later for DC depletion and Treg induction by flow cytometry. **(A)** Flow cytometry plots showing splenic DC populations in untreated, 250 μg DCR-5 and 250 μg isotype control treated mice. The $Lin^+/IAIE^+$ gate shown contains >98% $CD45R^+$ B cells (**Supplementary Figure 1A**). Graphs showing **(B)** frequency and **(C)** total numbers of DC populations in spleens of mice of all treatment groups. P <0.001 variance for treatment, 2-way ANOVA. **(D)** Combined data from four experiments showing frequency of DC populations in B6 mice treated with 250 μg DCR-5 or isotype control. Statistical comparison was determined using T-tests. *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$.

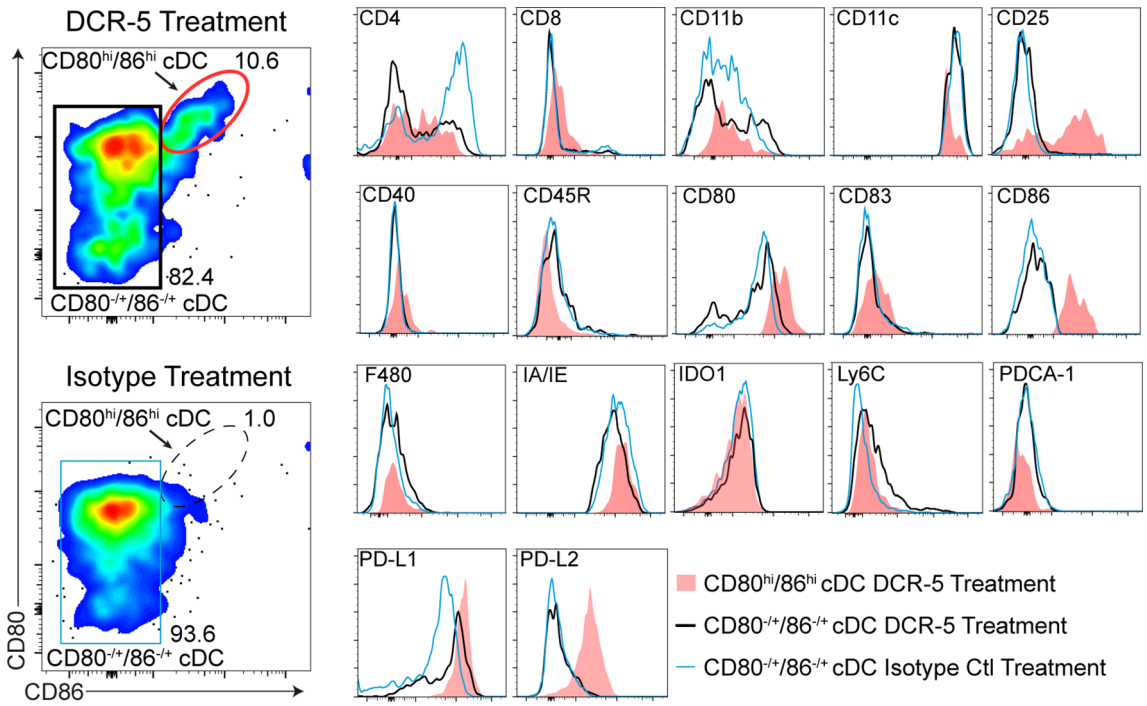

FIGURE 5 | Phenotype of CD80^{hi}CD86^{hi} DC population induced by DCR-5 *in vivo*. Flow cytometric expression of additional markers (histograms on right) on the populations gated in the left plots including the CD80^{hi}/CD86^{hi} cDC subset induced after 48 h i.p. treatment with 250 µg DCR-5 antibody (red oval gate) compared to remaining CD80$^{\pm}$/CD86$^{\pm}$ splenic cDC in DCR-5 (black rectangular gate) or isotype control (blue rectangular gate) treated mice.

in higher numbers, Tregs isolated from mice 48 h after DCR-5 treatment exhibited comparable capacity to prevent CD25^{-} T con proliferation than those from isotype treated mice when seeded at similar Treg : Tcon ratios in a Treg suppression assay (**Figure 6G**).

Correlation analyses of B6 mice treated *in vivo* with different doses of DCR-5 demonstrated significant negative correlations ($p < 0.0001$, Pearson coefficient correlation) between total cDC vs. CD80^{hi}/86^{hi} cDC (**Figure 6H**) and total cDC vs. Treg (**Figure 6I**), while a significant positive correlation was observed between CD80^{hi}/86^{hi} cDC vs. Treg ($p < 0.0001$, **Figure 6J**). These indicate an association between these parameters.

The concentration of the DCR-5 antibody was measured in the serum of B6 mice at 4 h, 2 d, 4 d and 7 d after 150 µg i.p. injection (**Figure 7A**). An initial serum concentration of 48 µg/ml at 4 h post-dose, decreased to approximately half this level between 2 and 4 d, and was no longer detectable at 7 d. Reduction of splenic cDC, particularly CD8^{+} cDC, was seen after 2 d DCR-5 treatment, but returned to numbers similar to untreated mice after 4 d (**Figure 7B**). However, a decrease in CD83^{+} cDC were still seen up to 4 d. Concomitantly, the CD80^{hi}/86^{hi} DC population induced by DCR-5 increased after 2 d but returned to near baseline levels by 4 d (**Supplementary Figure 3E**). Increases in splenic Treg numbers were still observed 7 d after DCR-5 treatment, however their proportion compared to conventional CD4^{+} T cells was diminished over 4–7 d post-treatment due to the increase in non-Treg (**Figures 7C–E**). In conclusion, *in vivo* DCR-5 treatment targets cDC populations, decreasing CD8^{+} and CD83^{+} subsets and induces maturation of a CD80^{hi}/86^{hi} DCreg-like population, resulting in an increase of Treg.

DCR-5 Can Induce DCreg Phenotype on Purified FL-DC Through Binding to CD83

To determine whether binding of DCR-5 to CD83 on DC was sufficient to induce a DCreg phenotype in the absence of other immune cells that mediate cytotoxicity, we generated a population of FL-DC from B6 bone marrow (consisting of >90% cDC and pDC). These were treated with DCR-5 or isotype control antibodies overnight and assessed for upregulation of DC maturation markers. While DCR-5 treatment caused notable changes to the surface phenotype of cDC but not pDC from these cultures, their proportions were not affected (**Figures 8A, B**). DCR-5 treatment of FL-DC in the absence of other cells led to most cDC expressing CD83 compared to isotype and untreated counterparts where some CD83$^{-/lo}$ cDC were present; although a proportion of control treated FL-DC expressed higher levels of CD83 than DCR-5 treated cDC (**Figure 8B**). Akin to observations in MLR and the *in vivo* experiments, DCR-5 treatment caused upregulation of CD80 and CD86 on cDC, which coincided with increases in CD25 and PD-L2 DCreg markers. Analogous DCR-5 mediated

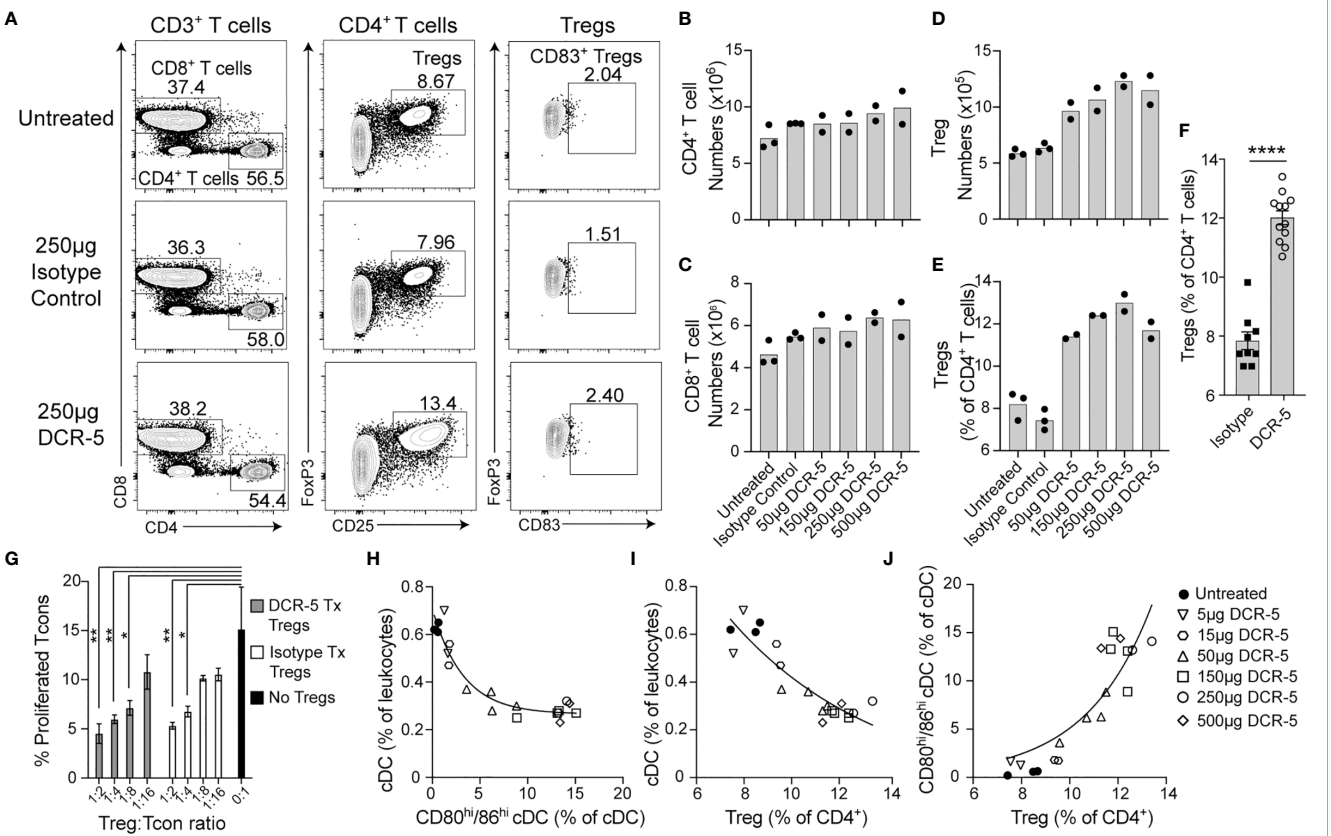

FIGURE 6 | *In vivo* treatment of mice with DCR-5 results in induction of Treg. B6 mice treated with a single i.p. injection of increasing doses of DCR-5 mAb or 250 µg rat IgG2a isotype control were analysed 48 h later for T cell subsets by flow cytometry. **(A)** Flow cytometry plots in one experiment showing T cell populations in spleens of untreated, 250 µg DCR-5 and isotype control mice. Total numbers of splenic **(B)** $CD4^+$ T cells, **(C)** $CD8^+$ T cells and **(D)** Tregs and **(E)** Treg as a percentage of $CD4^+$ T cells in mice treated with increasing DCR-5 doses. No significant difference for CD4 or CD8 T cell numbers and $p < 0.0001$ for Tregs numbers and proportion in combined DCR-5 versus isotype treated or untreated mice (one-way ANOVA). **(F)** Combined data from five experiments showing increase in Treg (as percentage of $CD4^+$ T cells) in mice treated with 150–250 µg DCR-5 or isotype control. Statistical comparison was determined using a T-test. ****$p < 0.0001$. **(G)** Treg suppression assay showing proliferation of CFSE labeled $CD45.1^+CD4^+$ Tcon purified from B6.*Ptprc*^{a} mouse spleens cocultured with anti-CD3/28 beads and the indicated ratios of $CD4^+CD25^+$ Tregs isolated from spleens of B6 mice after 48h treatment with 150 µg DCR-5 or isotype control i.p. All groups (n = 3) compared to Tcon only group by one-way ANOVA. *$p < 0.05$ and **$p < 0.01$. Correlation of **(H)** total cDC vs. $CD80^{hi}/86^{hi}$ cDC, **(I)** total cDC vs. Treg and **(J)** $CD80^{hi}/86^{hi}$ cDC vs. Treg frequencies in spleens of DCR-5 treated mice. All correlations were $p < 0.0001$ (Pearson correlation coefficient). Non-linear regression exponential curve fits shown.

induction of these markers were seen in $CD11b^+$ and $CD24^+$ subsets corresponding to $CD8^-$ and $CD8^+$ cDC (data not shown). Intracellular IDO1 expression was significantly increased by DCR-5 in both subsets (**Figures 8C, D**). Secretion of IL-10 was upregulated by exposure of FL-DC to DCR-5 in the absence or presence of maturation signals by LPS (**Figure 8E**). When DCR-5 treated FL-DC were washed and re-cultured with allogeneic BALB/c T cells, we saw a significantly increased capacity to induce Treg compared to control treated FL-DC, consistent with DCreg function (**Figures 8F, G**). This was not due to greater T cell activation as no change was observed in the percentage of activated ($CD25^+$ $FoxP3^-$) or proliferated $CD4^+$ T cells in the cultures (**Figures 8H, I**). These findings suggest that DCR-5 binding to CD83 on DC can directly induce maturation of DCreg which efficiently produce Tregs.

Optimized Dose of DCR-5 Reduces Severity of CIA

To determine whether anti-CD83 induced changes in the studies above would provide beneficial responses relevant for the treatment of autoimmune diseases, we assessed whether DCR-5 could reduce the severity of CIA developed by DBA/1 mice using the treatment regimens outlined in **Figure 9A**. Groups treated with different doses of DCR-5 (2, 6, and 10mg/kg) every three days throughout the study were compared to untreated, isotype control treated (10 mg/kg) and dexamethasone (0.2 mg/kg) treated groups. Weights were similar between DCR-5, isotype control or untreated groups through the study (**Supplementary Figure 4A**). However, mice treated with 2 and 6 mg/kg DCR-5 developed significantly lower clinical arthritic paw scores compared to mice treated with the isotype control or untreated animals (**Figures 9B, C**). The protective effect was lost in

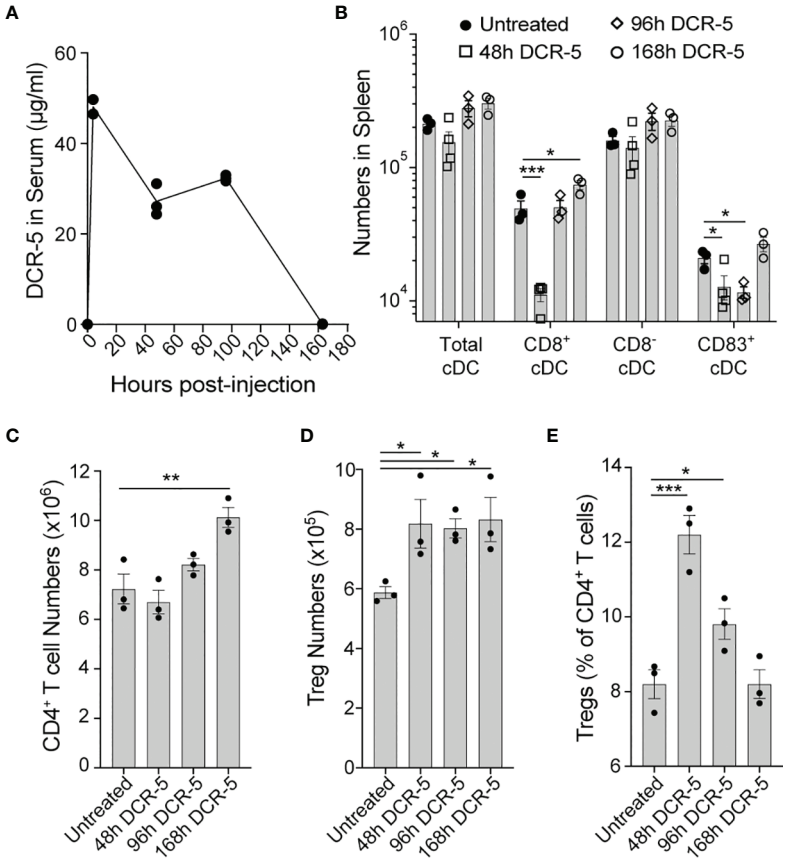

FIGURE 7 | Longevity of *in vivo* DCR-5 effects on DC and Treg. B6 mice were treated with a single i.p. injection of 150 µg of DCR-5 mAb. At the indicated timepoints post-injection, analysis of **(A)** DCR-5 concentration in serum by ELISA and total numbers of splenic **(B)** DC populations, **(C)** $CD4^+$ T cells, **(D)** Treg and **(E)** Treg as a percentage of $CD4^+$ T cells by flow cytometry was performed. Statistical comparison to untreated group was determined using one way ANOVA. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

the 10 mg/kg DCR-5 treated group. Consistently, 6 mg/kg DCR-5 treatment resulted in a significant decrease in serum anti-collagen antibodies compared to the isotype control and untreated groups, with a more modest decrease in 2 mg/kg and no effect from 10 mg/kg treatment (**Figure 9D**). Histopathological analysis of knees and ankles was performed on the isotype control and 6 mg/kg doses and showed significant amelioration of inflammation, pannus, cartilage damage, bone resorption, and periosteal bone formation from 6 mg/kg DCR-5 treatment compared to the isotype control (**Figures 9E, F** and **Supplementary Figure 4B**). Treatment with DCR-5 up to 6 mg/kg was therefore effective in alleviating disease in the CIA model.

DCR-5 Treatment Depleted $CD83^+$ DC and Induced Treg in CIA Model

Increased immunohistological CD83 staining in areas of lymphoid follicles that co-stain with CD11c (marking DC), but not CD45R (marking B cells), was observed in spleen sections of untreated DBA/1 mice euthanized at different timepoints during CIA development (**Figure 10A**). Analysis of spleen sections from 6 mg/kg DCR-5 treated compared to isotype control animals at the end of the CIA model (d36) revealed a clear decrease in CD11c and CD83 staining within follicles, indicative of specific depletion of DC (**Figure 10B**). Depletion of $CD83^+$ DC was less evident in spleens of 10 mg/kg DCR-5 treated mice. A similar decrease in CD11c staining in splenic follicles of 6 mg/kg DCR-5 treated mice was observed using immunohistochemistry methods (**Figure 10C**). DC depletion was not as marked in spleens of 10 mg/kg DCR-5 treated mice. In contrast, FoxP3 staining was increased in adjacent sections of 6 mg/kg DCR-5 treated mice compared to isotype control mice, indicating Treg induction (**Figure 10C**). Interestingly, despite poor disease control, limited FoxP3 staining was also induced in spleens of the 10 mg/kg DCR-5 group. Protection from arthritis in mice treated with 6 mg/kg DCR-5 is, therefore, associated with depletion of DC and induction of Treg in spleens, with these effects being less marked in 10 mg/kg DCR-5 treated mice.

Anti-Drug Antibodies (ADA) Elicited by DCR-5

To examine whether disparity in efficacy of DCR-5 at 6 and 10 mg/kg doses was caused by differences in anti-drug antibodies elicited by the treatments, we examined the sera collected at the end of the study (d36) from CIA groups for anti-DCR-5 antibodies *via* an anti-DCR-5 ELISA (**Supplementary Figure 4C**). High levels of anti-DCR-5 antibodies were

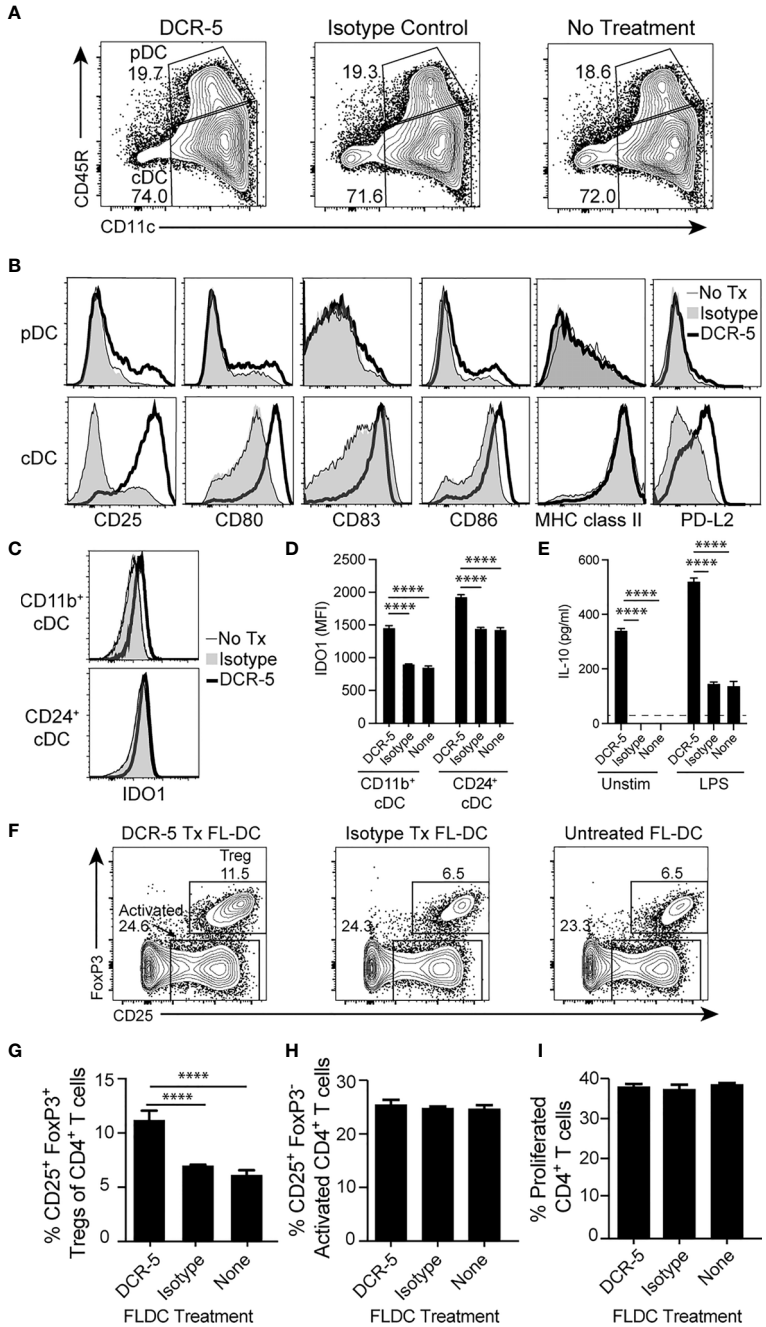

FIGURE 8 | DCR-5 binding to FL-DC population induces DCReg. **(A)** Flow cytometry plots showing gating of cultured B6 FL-DC cDC and pDC subsets after overnight culture with 20 µg/ml DCR-5, isotype control or no treatment and **(B)** histograms comparing their expression of maturation markers. **(C)** Representative histograms and **(D)** graph displaying flow cytometric intracellular expression (MFI) of IDO1 in FL-DC cDC subsets treated as above (n = 3). **(E)** Concentration of IL-10 in supernatants (n = 3) of FL-DC cultured for 48 h with 20 µg/ml DCR-5, isotype control or no antibody in the absence or presence of LPS stimulation. Dotted line shows limit of detection. **(F)** Representative contour plots and **(G)** graph (n = 3) of H-2K^{d+}CD4^{+}CD25^{+}FoxP3^{+} Treg generated from purified naïve BALB/c splenic T cells cultured at an 8:1 ratio for 3 days with washed B6 FL-DC after overnight treatment with DCR-5, isotype control or no treatment. **(H)** Graphs comparing CD25^{+} FoxP3^{-} T cells and **(I)** proliferated CFSE labeled CD4^{+} T cells in the same experiment. All groups compared by one-way ANOVA. ****p < 0.0001.

detected in the sera from 2, 6, and 10 mg/kg DCR-5 treated animals by d36 with no significant differences between the groups. Spiking the DCR-5 treated mouse serum samples with DCR-5, reduced binding in the ELISA indicative of anti-DCR-5 specificity. Interestingly, despite sharing immunoglobulin constant regions with DCR-5, treatment with 10 mg/kg isotype control did not stimulate measurable production of antibodies capable of binding to DCR-5.

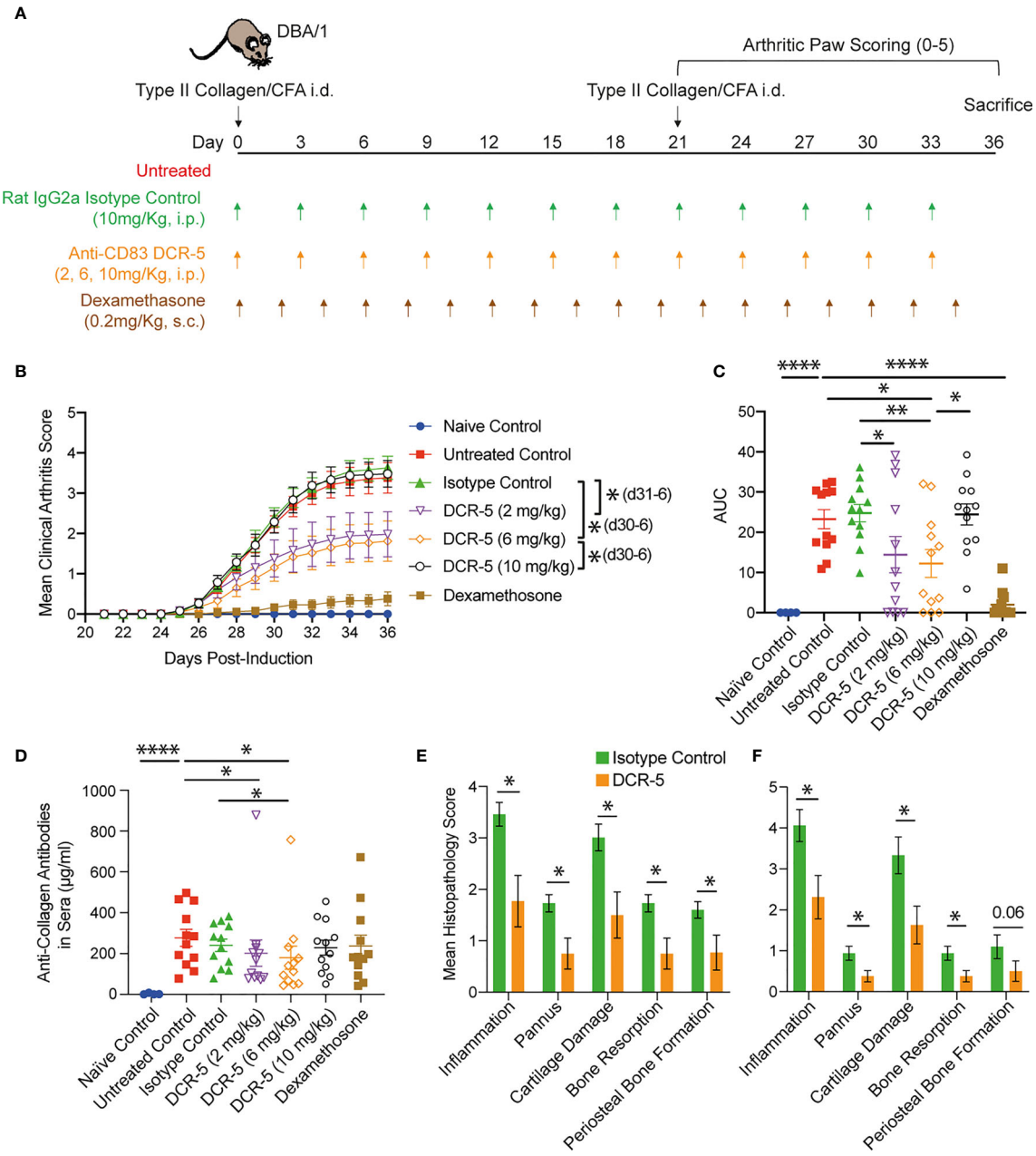

FIGURE 9 | Assessment of DCR-5 efficacy in CIA model. **(A)** Schema of CIA model and treatment regimes in five cohorts of 12 DBA/1 female mice. An additional four mice were not immunized with type II collagen in CFA as a naïve control cohort. **(B)** Mean arthritis clinical score per paw over time and **(C)** area under the curve (AUC) for each individual mouse (lines mark mean). Statistically significant differences at individual timepoints (in parentheses) and AUC were performed using one-way ANOVA. Significantly different timepoints outlined in parentheses. **(D)** Anti-collagen antibodies in serum at end of experiment were determined by ELISA. Statistical comparison of samples was determined by one-way ANOVA. **(E)** Mean paw and **(F)** knee score for each histopathology parameter in 6 mg/kg DCR-5 group versus the isotype control treated group at sacrifice. Statistical comparison of samples was determined by Mann–Whitney test. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

DISCUSSION

Targeting mature DC for depletion with anti-CD83 antibodies is a unique strategy for achieving specific immune modulation. Depletion of maturing DC prevents the ability to prime an effector T cell response while retaining the immature ($CD83^-$) DC that promotes T cell self-tolerance. As T cells are not the prime target, this treatment retains memory T cells specific for viral or tumor antigens, which are less reliant on DC for activation while accessory APCs such as B cells, macrophages, and non-haematopoietic cells can still activate an effector response (31, 32). Preserving memory T cells would provide a

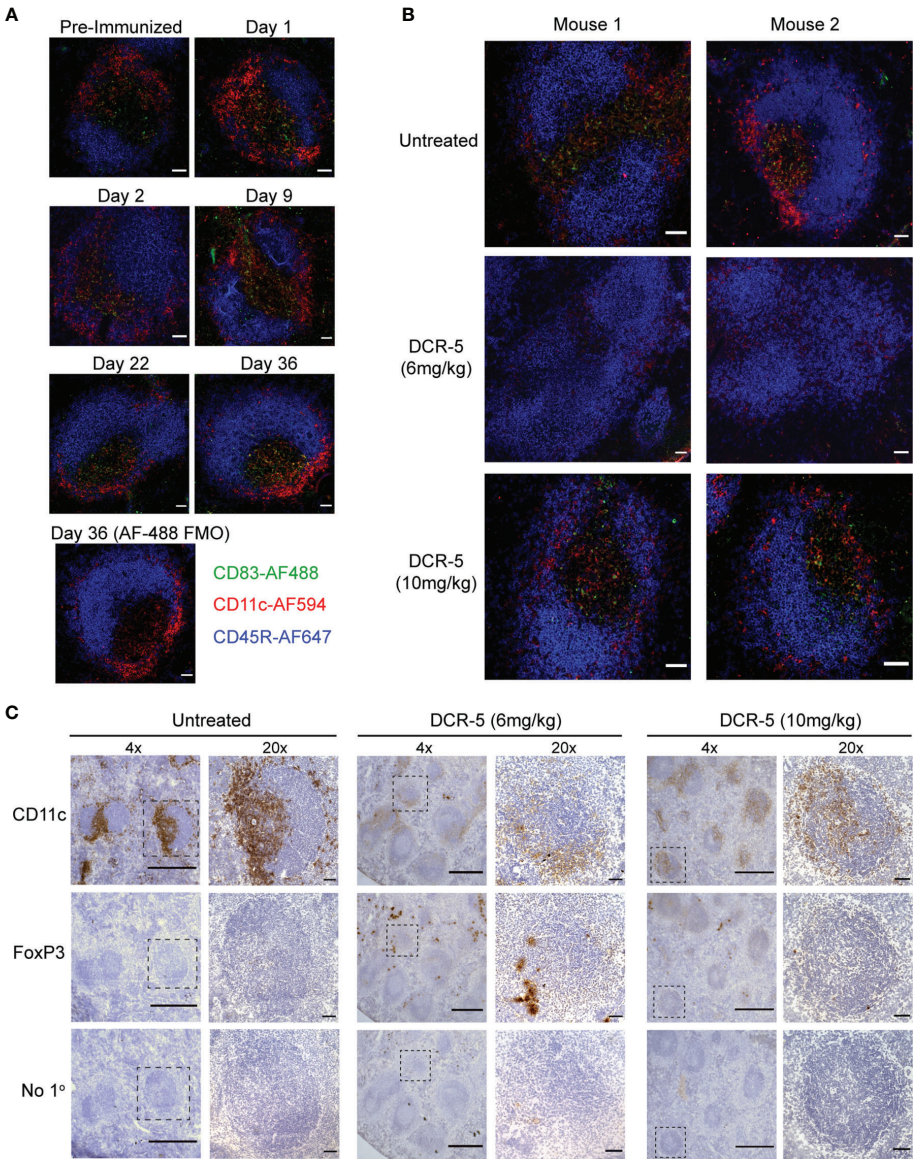

FIGURE 10 | DCR-5 treatment decreases CD11c staining and increases FoxP3 staining in spleens of CIA mice. **(A, B)** Immunofluorescence histology of splenic follicles showing CD83 detected with biotinylated Michel-19 clone together with CD11c and B220 marking DC and B cells, respectively. **(A)** Shows representative ×20 images from one of two DBA/1 mice euthanized pre-immunization or on the indicated days post-immunization of collagen. A control stain omitting the anti-CD83 primary antibody but including the Streptavidin-AF488 secondary is shown. Scale bars mark 50 µm. **(B)** Shows representative ×20 images of splenic follicles from two of four mice from untreated, 6 mg/kg or 10 mg/kg DCR-5 treated groups described in **Figure 9**. Scale bars mark 50 µm **(C)** Immunohistochemistry of adjacent spleen sections stained with biotinylated CD11c, FoxP3 antibodies or secondary streptavidin-HRP only. Representative ×4 image and marked inset at ×20 from one of four mice in untreated, 6 and 10 mg/kg DCR-5 treated groups are shown. Scale bars mark 500 and 50 µm in ×4 and ×20 photographs, respectively.

clinical advantage to patients by maintaining some immunity, preserving childhood vaccination responses, and limiting immune suppression.

While our human anti-human CD83 mAb 3C12C has shown the ability to deplete mature cDC and limit T cell responses in *in vitro* MLR experiments and xenogeneic graft-versus-host disease models (21), the ability to test the efficacy of this reagent in other inflammatory conditions such as autoimmune disease was limited by the dearth of animal models available to test human antibodies. The DCR-3 and DCR-5 rat mAbs were produced to mimic anti-CD83 mediated immune suppression in mice, to better understand its mechanism of action and test further indications for this therapeutic strategy in the plethora of inflammatory disease models developed in this species.

DCR-3 and DCR-5 exhibited greatest binding to the surface of mature cDC following activation, with less binding seen on activated B cells and other APC. However, DCR-3 and DCR-5 showed low levels of binding to CD83 on activated Treg compared

to the Michel-19 antibody. While this may be attributed to differences in affinity, the similar degree of binding by the antibodies to CD83 protein by ELISA and on the DC surface by flow cytometry would suggest that different CD83 isoforms on distinct cell populations contribute to the binding disparity. This was corroborated in our immunoprecipitation studies with DCR-5 and Michel-19, where common, but also distinct isoforms of CD83 were detected on the surface of mouse DC compared to B cells. Moreover, in our human studies (16), the HB15a mouse anti-human CD83 mAb clone bound preferentially to cell surface CD83 and less to intracellular CD83, with the reverse noted for the HB15e clone. MLR assays demonstrated that the DCR-5 IgG2a mAb had the ability to deplete $CD83^+$ cDC and inhibit T cell proliferation, while the DCR-3 IgG2b mAb did not. While rat IgG2a antibodies are more effective at activating rat NK cell ADCC than rat IgG2b (33, 34), in mice, rat IgG2b showed significantly greater capacity to induce ADCC than rat IgG2a (35). The lack of functional activity by DCR-3 is, therefore, surprising, and again highlights the importance of the CD83 epitope targeted for functional activity.

cDC that were not depleted by DCR-5 in the MLR assay expressed low to absent levels of CD83 but upregulated costimulatory molecules CD80 and CD86 together with the regulatory molecules PD-L2, CD25 and the IDO1 enzyme, indicative of DCreg maturation (4, 30, 36, 37). In addition, significant increases in IL-10 production and inhibition of IL-2 production were observed in DCR-5 treated MLR cultures. IL-10 production was induced in DC and Tcon in MLR. This is most likely due to direct and indirect effects of DCR-5, respectively, given that DC and not T cells were found to express CD83 in these cultures. DCR-5 was also shown to induce IL-10 production by FL-DC cultures. IL-10 is an important immunoregulatory cytokine that induces, and is produced by, DCreg and Treg (4, 38). CD25 expressed on the surface (and released in soluble form) by DCreg and also Treg acts to sequester available IL-2 to suppress conventional T cell proliferation (30, 38, 39). Expression of PD-L1 and PD-L2 by DCreg can induce deletion, anergy or Treg differentiation of interacting T cells expressing PD-1 (4). DCreg production of kynurenine, produced by tryptophan metabolism *via* the IDO1 enzyme, is a potent inducer of Treg differentiation upon binding to the aryl hydrocarbon receptor on T cells (4, 37, 40).

In vivo studies provided further insight into the function of DCR-5. I.p. injection of DCR-5 into mice showed that the DCR-5 antibody had the capacity to deplete splenic $CD83^{hi}MHCII^{hi}$ cDC populations *in vivo*. Cross-presenting $CD8^+$ cDC were particularly susceptible to DCR-5 depletion *in vivo* compared to $CD8^-$ cDC. While higher expression of CD83 was seen in LPS and CpG stimulated $CD8^+$ DC compared to $CD8^-$ cDC, the majority of $CD8^+$ cDC did not express CD83 in untreated or isotype treated mice. It is possible that exposure to DCR-5 may cause upregulation of CD83 in $CD8^+$ cDC. Akin to the MLR, DCR-5 induced the population of $CD83^{lo}$ $CD80^{hi}/86^{hi}$ cDC *in vivo* that upregulated DCreg markers including CD25, PD-L2 and PD-L1. The correlation found between the $CD80^{hi}/86^{hi}$ cDC population and Treg in DCR-5 injected mice was indicative of DCreg. We found that induction of a $CD80^{hi}/86^{hi}$ PD-$L2^+CD25^+IDO1^+$ phenotype with enhanced IL-10 producing capacity reminiscent of DCreg could be achieved by culturing DCR-5 with FL-DC alone. Moreover, this population had a heightened capacity to promote Treg differentiation, confirming DCreg function. Therefore, in addition to depletion of cDC, DCR-5 can drive DC maturation to a regulatory phenotype *via* binding to CD83. A previous study by Bates et al. (41) showed that ligation of membrane CD83 on human monocyte derived DC by anti-CD83 antibodies could mimic the immunoregulatory signals mediated by soluble CD83. Soluble CD83 can bind homotypically to membrane CD83 on DC resulting in inhibition of the p38 MAPK pathway (41) and induction of DCreg (42–46). While anti-CD83 antibodies have the potential to neutralise soluble CD83 regulatory activity (22), our data indicates that this may be compensated by antibody binding to membrane CD83. DCR-5 mediated apoptosis of mature DC by ADCC also has the potential to induce a DCreg phenotype when detected by bystander DC (47). This would fit with the negative correlation observed between the extent of DCR-5 mediated DC depletion and induction of the $CD80^{hi}/86^{hi}$ DC population. The extent to which these or other mechanisms contribute to DCreg generation by anti-CD83 mAb will be important to determine.

The relevance of our findings to human disease remains to be tested. In mice, immune populations expressing low levels of surface CD83 upon activation, namely, B cells, pDC, and Treg seem to be spared from DCR-5 mediated depletion. In contrast, the 3C12C human therapeutic mAb, which targets human and nonhuman primate CD83 has the capacity to deplete activated B cells both in human PBMC xenogeneic mouse models and non-human primates (22, 48). Unlike 3C12C that underwent affinity maturation using a light chain shuffling procedure (21) to select an antibody with higher affinity for human CD83, DCR-5 has not undergone this procedure and displays a 500-fold lower affinity for mouse CD83. This can contribute to their targeting differences. Despite reported differences between CD83 on T cells between mice and humans, neither 3C12C or DCR-5 antibody substantially depletes T cells, including Treg. While human Treg express minimal surface CD83 (16, 49), surface CD83 is found on a proportion of activated conventional T cells in humans and mice (29, 49–52). DCR-5 did not reduce conventional $CD4^+$ or $CD8^+$ T cells when injected into naïve mice. The lack of depletion is consistent with either the paucity of activated T cells in naïve mice or expression of a different isoform of CD83 on activated T cells. CD83 was not detected on T cells undergoing allogeneic proliferation in our MLR assay nor did we see marked CD83 expression on non-Treg T cells after CD3/CD28 activation. It is important to note that conventional T cells were not reduced post DCR-5 treatment in mice, indicating that the antibody is not likely to target $CD83^+$ thymic epithelial cells, which are essential for positive selection of T cells (18, 53).

DC in RA patients are central to the activation of autoreactive $CD4^+$ and $CD8^+$ T cells that drive effector mechanisms including autoantibody production, and for the recruitment of other inflammatory cells that induce joint pathology (9, 10). In mice,

administration of mature DC pulsed with collagen can initiate arthritis, suggesting that these cells are sufficient to drive disease pathogenesis (54). Alternately, administration of autoantigen presenting DC that are prevented from undergoing maturation, or differentiated into a regulatory phenotype, provide significant protection from arthritis (11–13). The main mechanism underlying this protection was the ability of transfused tolerogenic DC to enhance generation of Treg, which are potent at countering arthritogenic autoimmunity. Our study showed that DCR-5 treatment could deplete mature $CD83^+$ DC and augment the number of Treg in the CIA model, mechanisms that are likely to be essential to explaining our observations of reduced anti-collagen antibody titers, improved clinical scores, and preserved joint structure. Interestingly, the effect appeared to be dose dependent with up to 6 mg/kg dose providing optimal disease protection, while the higher 10 mg/kg dose was ineffective. Examination of spleens from treated animals indicated that the 10 mg/kg DCR-5 dose did exhibit some capacity to deplete DC and induce Treg in mice, but this was not as marked as in spleens from mice receiving the 6 mg/kg dose. One reason for the difference may be the onset and presence of ADA, as rat IgG2a antibodies can be highly immunogenic in mice, particularly when targeted to DC (55). All DCR-5 regimens induced high titers of ADA in serum by the end of the model. In most cases, higher doses of cross-species mAb in pre-clinical murine models result in higher risk of ADA (56, 57). Elevated levels of drug-ADA immunocomplexes also have the capacity to drive T cell independent B cell activation, further enhancing the ADA response (58, 59). It is conceivable that in our study the higher 10 mg/kg DCR-5 dose generates higher titers of ADA and immunocomplexes at earlier timepoints during the disease model, thereby shortening the therapeutic window. The 6 mg/kg dose could provide a more optimal balance of drug efficacy and ADA generation. The potential for ADA is reduced when treating humans with human mAb, however, assessment of ADA will be necessary when evaluating the 3C12C mAb in clinical studies. Another possibility for the lack of efficacy in the 10 mg/kg dose is that that higher doses of DCR-5 could target cells with more limited CD83 expression, namely, DCreg and Treg, leading to depletion of these cells and resulting in no observable pharmacodynamic effect. Our preliminary DCR-5 dose titration study in B6 mice did not reveal dose dependent differences in the type of immune populations that were depleted (e.g., B cells or pDC) or differences in Treg induction using higher doses (e.g., 250–500 μg). It should be noted though that this dose titration study administered a single dose under non-inflammatory conditions. Careful consideration of dose will be required to achieve optimal anti-CD83 immune modulation in patients.

In conclusion, we show here for the first time that depletion of mature DC by ligation of CD83 offers a unique mechanism for immunomodulation (**Figure 11**). The mechanism appears to involve the ability of the anti-CD83 antibody to deplete $CD83^{hi}$ DC, reducing antigen presentation by activated mature DC, upregulation of DCreg and the subsequent enhanced development of Treg. By using the CIA model, we demonstrate that these properties translate to effectiveness in a model that fits the relevant biology and is a common animal model for

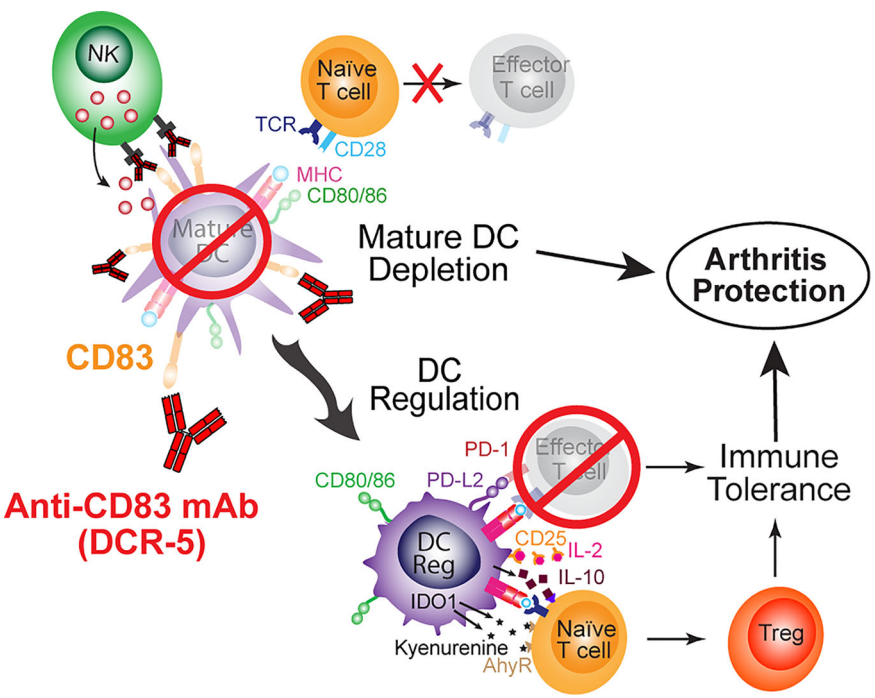

FIGURE 11 | Schematic of mechanisms of anti-CD83 mAb mediated immune suppression leading to protection from arthritis.

rheumatoid arthritis. The biology of anti-CD83 antibodies has potential in the treatment of autoimmune, inflammatory or transplant related diseases. Further animal models, including murine models using DCR-5 and nonhuman primate models using the human/primate antibody (3C12C) will address the pre-clinical and mechanistic studies to understand how this treatment could benefit patients with these diseases.

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

The animal studies were reviewed and approved by the Bolder Biopath Ethics Committee and the Sydney Local Health District Animal Welfare Committee.

AUTHOR CONTRIBUTIONS

PS designed research studies, conducted experiments, acquired, and analysed data and wrote the manuscript. FK, AR, W-HH, T-HL, and H-TC conducted experiments, acquired, and analyzed data. XJ, HR, and DB contributed to the design of research studies, analysis of data and reviewing the manuscript. GC contributed to the design of research studies, analysis of data and writing of the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.784528/full#supplementary-material

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Anti-Tumor Potency of Short-Term Interleukin-15 Dendritic Cells Is Potentiated by *In Situ* Silencing of Programmed-Death Ligands

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Dendritic cell (DC) vaccines have proven to be a valuable tool in cancer immune therapy. With several DC vaccines being currently tested in clinical trials, knowledge about their therapeutic value has been significantly increased in the past decade. Despite their established safety, it has become clear that objective clinical responses are not yet robust enough, requiring further optimization. Improvements of this advanced therapy medicinal product encompass, among others, regulating their immune stimulating capacity by *in situ* gene engineering, in addition to their implementation in combination therapy regimens. Previously, we have reported on a superior monocyte-derived DC preparation, including interleukin-15, pro-inflammatory cytokines and immunological danger signals in the culture process. These so-called IL-15 DCs have already proven to exhibit several favorable properties as cancer vaccine. Evolving research into mechanisms that could further modulate the immune response towards cancer, points to programmed death-1 as an important player that dampens anti-tumor immunity. Aiming at leveraging the immunogenicity of DC vaccines, we hypothesized that additional implementation of the inhibitory immune checkpoint molecules programmed death-ligand (PD-L)1 and PD-L2 in IL-15 DC vaccines would exhibit superior stimulatory potential. In this paper, we successfully implemented PD-L silencing at the monocyte stage in the 3-day IL-15 DC culture protocol resulting in substantial downregulation of both PD-L1 and PD-L2 to levels below 30%. Additionally, we validated that these DCs retain their specific characteristics, both at the level of phenotype and interferon gamma secretion. Evaluating their functional characteristics, we demonstrate that PD-L silencing does not affect the capacity to induce allogeneic proliferation. Ultimately designed to induce a durable tumor antigen-specific immune response, PD-L silenced IL-15 DCs were capable of surpassing PD-1-mediated inhibition by antigen-specific T cells. Further corroborating the superior potency of short-term IL-15 DCs, the combination of immune stimulatory components during DC differentiation and maturation with *in situ* checkpoint inhibition supports further clinical translation.

Keywords: dendritic cells, interleukin-15, programmed death (PD)-1, programmed death 1 ligand, siRNA, Wilms' tumor 1 (WT1)

INTRODUCTION

Ever since the introduction of dendritic cells (DCs) in the field of cancer immunotherapy (1), extensive research has been done to exploit this therapeutic modality (2–5). From early clinical trials, it is now generally accepted that DC vaccination is well tolerated and safe (6). While DC vaccination can elicit immune responses in many patients, objective clinical responses remain limited and prone to improvement. Our group previously reported the development of a novel DC type, by differentiating monocytes with granulocyte-macrophage colony stimulating factor (GM-CSF) complemented with the pleiotropic cytokine interleukin (IL)-15—instead of the more classically used IL-4—and a maturation cocktail including interferon (IFN)-γ, prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α and a Toll-like receptor (TLR) 7/8 agonist (7). In the past decade, we extensively researched this type of monocyte-derived DC (moDC), hereafter referred to as IL-15 DC, demonstrating its superior immunostimulatory capacity. First, we showed that IL-15 DCs are superior in terms of their capacity to induce both T helper 1 and cytotoxic T-cell responses (7, 8) and to potentiate natural killer (NK) cell and gamma delta (γδ) T cell cytotoxicity (9–12). Moreover, IL-15 DCs have intrinsic cytotoxic properties, allowing them to be listed as 'killer DCs' (8, 13). Interestingly, IL-15 DCs are able to secrete the immune regulatory cytokines IFN-γ and IL-15 and granzyme B, which contribute to their direct cytotoxic efficacy (8).

In our previous clinical trials with conventional IL-4 DC vaccines [NCT01686334 and NCT00834002 for acute myeloid leukemia (AML)] we have observed favorable objective responses. More clinical research with these IL-4 DCs is currently being conducted by our group (NCT02649829 for mesothelioma, NCT02649582 for glioblastoma, NCT01291420 for solid tumors). IL-4 DC-vaccination prolonged relapse-free survival in AML patients; however, not all IL-4 DC-vaccinated patients responded equally well, and the majority of responders eventually relapsed (14). This disparity in responses seen in IL-4 DC-vaccinated patients and the suboptimal efficacy of the treatment can be partially explained by the presence of costimulatory and inhibitory signals, whose balance are involved in determining the strength of an immune response. The presence of inhibitory immune checkpoints and/or their ligands on the surface of DCs, has been demonstrated *in vivo* in mice to hamper their stimulatory capacity towards immune effector cells. Indeed, programmed death 1 (PD-1) ligand 1 (PD-L1) is highly expressed on DCs and blockade of PD-L1 can reactivate tumor-infiltrating T cells (15). Merging the evolving science on the role of PD-1/PD-L signaling in DC-mediated immunity and the pursuit of developing highly immunogenic DC vaccines, a new therapeutic approach to interfere with the PD-1/PD-L pathway has been introduced by Dolstra et al. They showed that conventional IL-4 DCs express high levels of the co-inhibitory molecules PD-L1 and PD-L2, which can be downregulated following transfection with specific short interfering or silencing RNA (siRNA) (16–18). These PD-L-silenced antigen-loaded DCs superiorly boosted *ex vivo* and *in vivo* minor histocompatibility antigen-specific T cell responses from leukemia patients (17). The safety and feasibility of these DCs to induce more robust clinical responses is currently being investigated (NCT02528682). Given this successful improvement of the stimulatory capacity of IL4 DCs, we subsequently showed that combining *in situ* downregulation of PD-L1 and PD-L2 with introduction of interleukin-15 transpresentation tools could further potentiate tumor-reactive T-cell expansion (19).

Extending the preclinical development of short-term IL-15 DCs as therapeutic cancer vaccine, the aim of this study was to confirm the added value of *in situ* PD-L1 and PD-L2 silencing of IL-15 DCs, harnessed with a unique immune-stimulating profile that significantly outperforms IL-4 DC-mediated *in vitro* anti-tumor activity (7–13). Following successful development and qualification of a PD-L silencing protocol for 3-day cultured IL-15 DCs, their functional capacity to regulate proliferation and immunostimulatory cytokine production by allogeneic and autologous primary lymphocytes was evaluated. Ultimately, by targeting the tumor-associated antigen Wilms' tumor-1 (WT1), the tumor-antigen specificity was assessed by demonstrating a superior antigen-specific T cell stimulating capacity of PD-L-silenced IL-15 DCs. The data presented here provide a rationale for implementing PD-L silenced IL-15 DCs as next generation anticancer vaccines in upcoming clinical trials.

MATERIALS AND METHODS

Ethics Statement and Primary Cell Material

This study was approved by the Ethics Committee of the University Hospital Antwerp/University of Antwerp (Antwerp, Belgium) under reference number 16/10/123. Peripheral blood mononuclear cells (PBMCs) were isolated from anonymous donor buffy coats provided by the Blood Service of the Flemish Red Cross (Mechelen, Belgium) by means of Ficoll density gradient centrifugation. Positively selected $CD14^+$ monocytes were isolated from PBMCs by means of $CD14^+$ magnetic microbeads for magnetic-activated cell sorting (MACS; Miltenyi Biotec, Leiden, The Netherlands) and were freshly cultured into IL-15DCs (*vide infra*). $CD14^-$ peripheral blood lymphocytes (PBLs) were cryopreserved or used for isolation of NK cells by negative immunoselection using the NK cell isolation kit (Miltenyi Biotec). PBLs and NK cells were cryopreserved at a maximum cell concentration of $100x10^6$/mL in 1 mL fetal bovine serum (FBS, Life Technologies, Merelbeke, Belgium) supplemented with 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Diegem, Belgium) per cryovial.

Cell Lines

The HLA-A*02:01 positive, WT1-negative multiple myeloma cell line U266 was kindly provided by Prof. W. Germeraad (Maastricht University Medical Center, Maastricht, The Netherlands). T cell receptor (TCR)-deficient Jurkat J76.7 cells transduced to express eGFP after activation of a specific introduced TCR, hereafter called 2D3 cells, were kindly provided by prof. H. Sugiyama (Osaka University Graduate School of Medicine, Osaka, Japan) under material transfer agreement (MTA)19-308. $PD\text{-}1^+$ 2D3 cells were stably transduced with PD-1 by prof. K. Breckpot (Free University Brussels, Brussels, Belgium) (20). All cell lines were maintained in

Roswell Park Memorial Institute medium (RPMI; ThermoFisher Scientific) supplemented with 10% FBS.

mRNAs and siRNAs

Codon-optimized Sig-DC-LAMP *WT1* mRNA encoding isoform D of WT1 (14) was used to transfect antigen-presenting cells. The human $WT1_{37\text{-}45}$-specific TCR gene was generated as described in (21). The coding sequence of PD-1 was cloned into the pST1 [RHAMM] vector using SpeI and XhoI cloning sites to produce [pST1 PDCD1 vector] (GeneArt, ThermoFisher Scientific). The original pST1 backbone vector was kindly provided by Dr. Ugur Sahin (Johannes-Gutenberg University, Mainz, Germany) (22) under MTA. Plasmid DNA was propagated as described in (21). Next, plasmids were linearized after the 120 bp poly(A) tail using SapI restriction enzyme (ThermoFisher Scientific). mRNA was synthesized from linearized plasmid template using an mMessage mMachine T7 *in vitro* transcription kit (ThermoFisher Scientific) according to the manufacturer's instructions. PD-L1 and PD-L2-targeting siRNAs as well as control luciferase-targeting siRNAs were produced as described in (17, 18) and were kindly delivered by Prof. H. Dolstra and Prof. W. Hobo (Radboud University medical center, Nijmegen, The Netherlands).

Generation of IL-15 DCs

The culture protocol of short-term IL-15 DCs was adapted from the previously described protocol (7) to include the disruption of newly synthesized PD-L1 and PD-L2 (**Supplementary Figure S1**). Isolated $CD14^+$ monocytes were transfected in GMP-ready serum-free phenol red-free X-VIVO15 medium (Lonza, Verviers, Belgium). For each transfection, optimized amounts of 2 µg siRNAs (luciferase (luci) or PD-L1/PD-L2 (PD-L), 2:1 ratio) were pre-incubated with 10 µL SAINT-RED (Synvolux, Leiden, The Netherlands) per mL transfection volume. After 15 minutes, 4.5–6.0 x 10^6 monocytes were resuspended in X-VIVO15 per mL transfection volume and transferred to a T75 culture flask (7 mL transfection volume) or T175 culture flask (15 mL transfection volume). For the non-transfected condition (no), neither siRNAs nor SAINT-RED was used. After 1 hour of lipofection at 37°C, differentiation medium was added to a total volume of 21 mL in T75 or 45 mL in T175 flasks, resulting in a cell culture density of 1.5–2 x 10^6 monocytes/mL. Differentiation medium was prepared for final culture medium (transfection volume + differentiation medium) concentrations of 800 IU/mL GM-CSF, 200 ng/mL IL-15 and 1% hAB serum (Life Technologies). After 48 h, a maturation cocktail containing 3 µg/mL TLR 7/8 ligand R-848 (Enzo Life Sciences, Antwerp, Belgium), 2.5 ng/mL TNF-α (Gentaur, Brussels, Belgium), 250 ng/mL IFN-γ (Immunotools, Friesoythe, Germany) and 1 µg/mL PGE2 (Prostin E2, Pfizer, Puurs, Belgium) was added for 16-20 hours (12). Mature PD-L-silenced IL-15 DCs were harvested and cryopreserved at 5-15 x 10^6 cells per vial in 70% X-VIVO15 medium, 20% hAB serum (Life Technologies) and 10% DMSO.

Membrane Phenotyping of IL-15 DCs

For membrane immunophenotyping of IL-15 DCs, FcRγIII receptors on IL-15 DCs were blocked using mouse gamma-globulins (Jackson Immunoresearch, Suffolk, UK). IL-15 DCs were characterized by immunofluorescent surface staining using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mABs) recognizing CD83, CD274 (PD-L1) and IL-15, phycoerythrin (PE)-conjugated mABs recognizing CD14, CD56, CD80 and CD273 (PD-L2) or PE-Cy7-conjugated mABs recognizing CD7 and CD86 (BD Biosciences, Erembodegem, Belgium; Invitrogen, Camarillo, CA, USA; R&D Systems, Minneapolis, MN, USA). Corresponding species- and isotype-matched antibodies were used as negative controls. Viability was determined using either propidium iodine (PI; Invitrogen) or 7-aminoactinomycin D (7-AAD; BD Biosciences). Samples were acquired on a CytoFLEX flow cytometer (Beckman Coulter, Suarlée, Belgium).

Electroporation

Cells were electroporated in a 4-mm cuvette (Cell Projects, Harrietsham, UK) in a Gene Pulser Xcell electroporator (Biorad, Temse, Belgium) using 1 µg mRNA per 10^6 cells. Fresh 5-10 x 10^6 IL-15 DCs were electroporated with *WT1* mRNA in 250 µL OptiMEM (ThermoFisher Scientific) with an exponential decay pulse (300V, 150 µF) (7). U266, used as control antigen presenting cells (APCs), were treated identically but electroporated using a time constant pulse (300V, 8 ms) (23). *WT1* mRNA transfection efficiency was determined 4 hours post electroporation by means of intracellular staining employing the eBioscience FoxP3/transcription factor intracellular staining buffer set (Invitrogen) and an anti-WT1 primary antibody (clone 6F-H2, Dako, Agilent, CA, US) as described previously (23). 10-20 x 10^6 $PD\text{-}1^+$ 2D3 cells were electroporated in 400 µL OptiMEM with *TCR* mRNA as described before (20). 20-40 x 10^6 thawed PBLs and purified NK cells were electroporated in 500 µL OptiMEM with *PD-1* mRNA using a square wave protocol (500V, 5 ms). TCR and PD-1 protein expression was evaluated 2 hours post electroporation by surface staining with pan-TCRαβ-PE (Miltenyi Biotec) and CD279-FITC (BD Biosciences), respectively. All acquisitions were performed on a CytoFLEX flow cytometer.

Peptide-Loading of IL-15 DCs

Alternatively to electroporating full-length *WT1* mRNA, IL-15 DCs were peptide-pulsed with $WT1_{37\text{-}45}$ peptide (VLDFAPPGA; JPT, Berlin, Germany). DCs were resuspended in serum-free RPMI medium at a concentration of 2 x 10^6 IL-15 DCs/mL in polypropylene tubes. 10 µg/mL of the peptide was added, and cells were incubated at room temperature on a horizontal tilting tube roller covered from direct light. After 1 hour, cells were washed and resuspended in RPMI supplemented with 10% FBS for use in functional assays.

Allogeneic Mixed Lymphocyte Reaction (Allo-MLR)

PBL from an allogeneic blood donor were thawed, transfected with *PD-1* mRNA (*vide supra*) or mock transfected (no mRNA), and stained with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 µM, Invitrogen) as previously described (8), prior to co-culture with IL-15 DCs at an APC : PBL ratio of 1:10.

Stimulation with phytohemagglutinin (PHA; 1μg/mL; Sigma-Aldrich, Overijse, Belgium) and IL-2 (20 IU/mL; Immunotools) served as a positive control (8). After 5 days, co-culture supernatant was collected and stored at -20°C until further use (ELISA, *vide infra*). Cell pellets were washed and stained with CD3-PerCP-Cy5.5, CD4-APC, CD8-Brilliant Violet 786, CD56-PE and CD279-Brilliant Violet 421 (all from BD Biosciences). Fixable Aqua dead cell stain (ThermoFisher Scientific) was used to discriminate between viable and dead cells. Samples were acquired on a FACSAria II flow cytometer (BD Biosciences). An example of the applied gating strategy is depicted in **Supplementary Figure 2**.

DC-Mediated NK Cell Activity Assay

To evaluate the NK cell-stimulating capacity of the PD-L-disrupted IL-15 DCs, DCs were co-cultured with autologous non-electroporated or *PD-1* mRNA-electroporated purified NK cells in 96-well round-bottom plates at a ratio of 1:1 (250.000 cells per cell type) in triplicate at 37°C. 24h and 48h cell-free culture supernatant was collected and stored at -20°C for assessment of NK cell-mediated IFN-γ secretion (*vide infra*).

WT1-Specific PD-1$^+$ 2D3 T Cell Assay

$WT1_{37-45}$ specific *TCR* mRNA-transfected PD-1^+ 2D3 cells (20) were co-cultured in 96-well plates with no/luci/PDL siRNA IL-15 DCs or U266 cells (as positive control) that were electroporated with *WT1* mRNA or peptide-pulsed with $WT1_{37-45}$ peptide or left unpulsed at a 2D3:APC ratio of 2:1 (100.000:50.000 cells per well). When indicated, $1x10^6$/mL 2D3 cells were pre-incubated for 1 hour with 15μg/mL anti-PD-1 antibody nivolumab (kindly provided by the pharmacy of the Antwerp University Hospital), prior to co-culture with APCs. After 16-hour co-culture at 37°C, supernatant was collected and stored at -20°C until further use. Cell pellets were stained with CD8-PE, washed, incubated for 10 minutes with 7-AAD and subsequently assessed for eGFP expression. Samples were acquired on a CytoFLEX flow cytometer. An example of the applied gating strategy is depicted in **Supplementary Figure 3**.

ELISA

Secretion of IFN-γ by IL-15 DCs or lymphocytes was determined in 24-hour washout supernatant or 5-day co-culture supernatant, respectively), using an enzyme-linked immunosorbent assay (ELISA; Peprotech, US) following the manufacturer's instructions. Samples were diluted when necessary to fit the standard curve of the kit with a detection limit of 16 pg/mL and top standard of 2000 pg/mL. Secretion of granzyme B by 2D3 cells and IL-15 DCs was determined in 16-hour co-culture supernatant or 48h monoculture washout supernatant using ELISA (R&D systems) following the manufacturer's instructions. A sample dilution of 1:8 was optimal to fit the standard curve of the kit (with a detection limit of 24.4 pg/mL and top standard of 2500 pg/mL) when analyzing the 2D3 coculture supernatant, while supernatant from monocultures was measured undiluted. For both ELISAs immunoluminescence was measured on a Victor 3 multilabel counter (Perkin Elmer).

Statistical Analysis

Data were statistically analyzed and represented with GraphPad Prism (version 9, CA, US). The data was checked for normal distribution and homogeneous variances. When data were normally distributed parametric analyses were performed. When normal distribution could not be confirmed, non-parametric analyses were performed. Data are expressed as mean ± standard error of mean (SEM). *P*-values smaller than 0.05 were considered statistically significant ($*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$).

RESULTS

Highly Efficient *In Situ* PD-L1/2 Silencing in Short-Term IL-15 DCs

Our group previously reported a protocol for silencing monocyte-derived IL-4 DCs before maturation, in which 40 μL of SAINT-RED per mL were used for transfecting siRNAs (19). However, we observed that this concentration of SAINT-RED resulted in low IL-15 DC viability with a very high experimental variability between replicates (62.3 ± 29.10 % PI$^-$ IL-15 DC; **Figure 1A**). Therefore, to determine non-cytotoxic concentrations of lipofectant for transfection of purified monocytes prior to differentiation into IL-15 DCs, we performed a titration of SAINT-RED and assessed the viability of mature IL-15 DCs at harvest (**Figure 1A**). Monocytes treated with 20 μL or 10 μL/mL SAINT-RED, could be robustly differentiated into highly viable mature IL-15 DCs (93.50 ± 0.40% PI$^-$ IL-15 DC and 93.50 ± 1.10% PI$^-$ IL-15 DC, respectively) similar to their non-transfected counterparts (90.25 ± 0.85% PI$^-$ IL-15 DC), whereas 30 μL/mL SAINT-RED seemed to have a cytotoxic effect at least in some donors (88.75 ± 6.15% PI$^-$ IL-15 DC), albeit lower than 40 μL/mL. Continuing with10 μL SAINT-RED per mL transfection volume, the ratio of siRNA to SAINT-RED was further optimized. The ratio of 2:1 PD-L1:PD-L2 siRNA was kept as described previously (19), but total amount of siRNA was further downscaled to 2 μg/mL transfection volume due to the toxic effect of high concentrations of siRNA (data not shown). Silencing efficiency of PD-L siRNA lipofection was evaluated by calculating the relative expression of PD-L1 and PD-L2 on mature IL-15 DCs of the PD-L siRNA condition in relation to the paired control luciferase siRNA condition (ΔMFI PD-L condition/ΔMFI luci condition, with ΔMFI = MFI of PD-L expression – MFI of matched isotype). Similar ΔMFI of PD-L1 surface expression were observed on non-lipofected IL-15 DCs and on control luci siRNA IL-15 DCs ($\Delta MFI_{PD\text{-}L1}$ 8057 ± 515.9 and 8495 ± 603.6 (n=48), respectively; **Figure 1B**, left panel). Equally, IL-15 DC PD-L2 surface expression on untreated IL-15 DCs was similar to that of IL-15 DCs treated with control luci siRNA ($\Delta MFI_{PD\text{-}L2}$ 908.2 ± 306.2 and 1159 ± 392.1, respectively; **Figure 1B**, right panel) (n=48). Surface expression of both ligands was efficiently disrupted in mature siRNA IL-15 DCs, with a $\Delta MFI_{PD\text{-}L1}$ of 1288 ± 84.8 (n=48; $p < 0.0001$ compared to no siRNA and luci siRNA IL-15 DCs) and $\Delta MFI_{PD\text{-}L2}$ of 194.0 ± 58.7 (n=48, $p = 0.0114$ compared

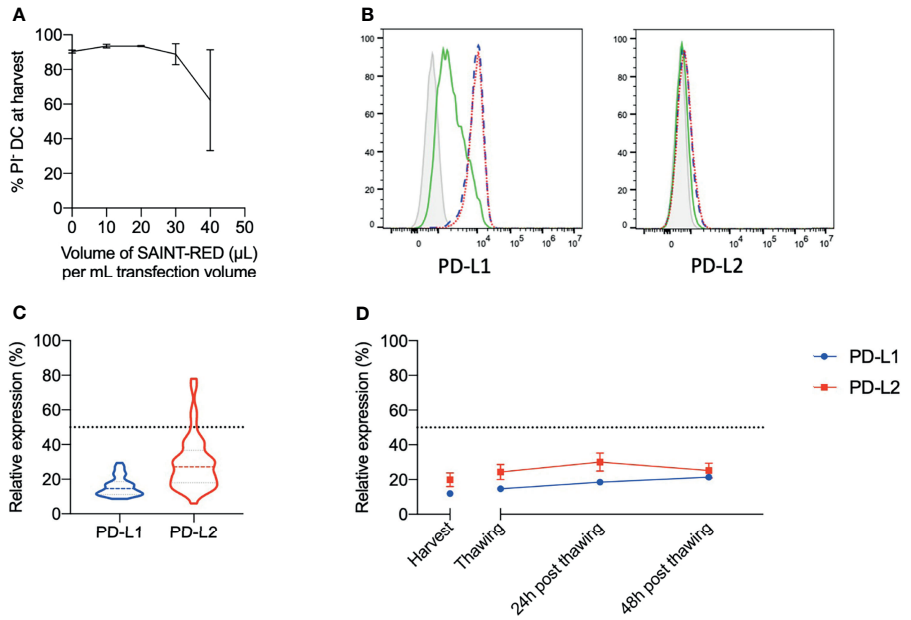

FIGURE 1 | Optimization of siRNA transfection protocol in monocytes and assessment of transfection efficiency assessed on mature IL-15 DCs. **(A)** The effect of increasing concentrations of SAINT-RED lipofectant (expressed in µL/mL transfection volume for 4.5-6.0 x 10^6 monocytes/mL) on mature IL-15 DC viability at harvest was assessed flow cytometrically with PI (n = 3). **(B)** Representative histogram overlays of the surface expression of PD-1 ligands PD-L1 and PD-L2 in mature IL-15 DCs at harvest from untreated (blue dashed line), luciferase siRNA-treated (red dotted line), and PD-L siRNA-treated (green line) monocytes. Grey filled line represents the corresponding isotypes. **(C)** Relative expression of PD-L1 and PD-L2 in mature PD-L siRNA IL-15 DCs compared to luci siRNA IL-15 DCs of PD-L1 and PD-L2 at harvest (n = 48). The horizontal dotted line indicates a cut-off value of 50% relative expression as indicator of successful silencing. **(D)** Kinetics of surface PD-L1 and PD-L2 relative expression on fresh mature IL-15 DCs at harvest and 1, 24 or 48 hours after thawing (n = 8). PD-L1, programmed death-1 ligand 1; PD-L2, programmed death-1 ligand 2.

to no siRNA and luci siRNA IL-15 DCs). These data are summarized in **Table 1**. Successful silencing was set at a threshold value of 50% reduction in relative expression of PD-L1 and PD-L2 (**Figures 1C, D**). As shown in **Figure 1C**, calculated relative expression levels were far below the set threshold of 50% silencing, with a relative expression of 15.94 ± 0.80% for PD-L1 and 29.47 ± 2.40% for PD-L2. Stable PD-L1 and PD-L2 siRNA-mediated silencing was confirmed after thawing matured IL-15 DCs (14.71 ± 0.66% and 24.36 ± 4.38% relative expression of PD-L1 and PD-L2, respectively) and at different time points after thawing, as demonstrated by the constant low relative expression below the 50% cut-off value (**Figure 1D**).

PD-L Silencing Has No Negative Impact on Mature IL-15 DCs Characteristics

Confirming the reproducibility of the optimized PD-L siRNA IL-15 DC culture protocol, comparable and robust monocyte-to-mature IL-15 DC yields were obtained for untreated (58.02 ± 1.79%), luci siRNA-treated (56.68 ± 1.78%), and PD-L siRNA-treated (59.90 ± 1.59%) IL-15 DCs at harvest (n = 40; **Figure 2A** and **Table 1**). Viability of mature IL-15 DCs at harvest was maximal for all conditions (93.66 ± 0.49%, 92.03 ± 0.81% and 93.47 ± 0.62% PI^- for untreated, luci siRNA and PD-L siRNA IL-15 DCs, respectively; **Figure 2B** and **Table 2**). To anticipate the effect of siRNA-mediated silencing on reconstitution of thawed IL-15 DCs for vaccine administration, the pre-to-post-cryopreservation recovery was assessed (**Figure 2C**). While only 70.64 ± 5.57% of untreated IL-15 DCs could be viably recovered after cryopreservation, better post-cryopreservation recoveries were achieved for luci siRNA (81.45 ± 7.69%) and PD-L siRNA IL-15 DCs (83.15 ± 7.07%). Phenotypically, expression levels of conventional DC maturation markers CD80, CD83 and CD86, and the prototypic IL-15 DC surface marker CD56, did not differ between the untreated and siRNA-treated IL-15 DCs (**Figure 2E**). In line with this, secretion levels of the immune-regulator IFN-γ, a key feature of IL-15 DCs, were

TABLE 1 | Summary of ΔMFI ($MFI_{PD\text{-}L}$ – $MFI_{isotype}$) of no siRNA IL-15 DCs (no), luciferase siRNA IL-15 DCs (luci) or PD-L siRNA IL-15 DCs (PD-L), n = 48.

	No	Luci	PD-L
PD-L1	8057 ± 515.9	8495 ± 603.6	1288 ± 84.8
PD-L2	908.2 ± 306.2	1159 ± 392.1	194 ± 58.7

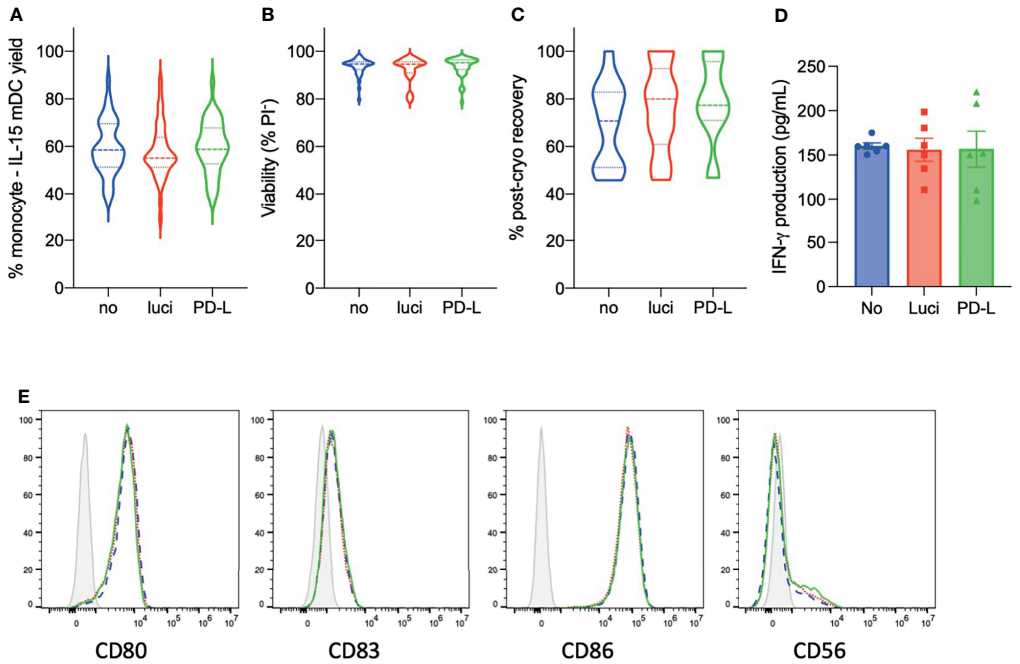

FIGURE 2 | Phenotypic characterization of PD-L silenced mature IL15 DCs. Violin plots of **(A)** monocyte-to-mature IL-15 DC yields (n = 44), **(B)** viability (% PI^-; n = 44) and **(C)** post-cryopreservation (post-cryo) recovery 15 to 30 min after thawing (n = 13) from untreated (no), control siRNA-treated (luci) and PD-L1/PD-L2 siRNA-treated (PD-L) IL-15 DCs at harvest. **(D)** Concentration of IFN-γ, an IL-15 DC hallmark, in 24-hour cell-free supernatants of IL-15 DC monocultures (100,000 cells; n = 6). **(E)** Representative histogram overlays of key surface markers of mature IL-15 DCs on no siRNA IL-15 DCs (blue dashed line) and PD-L siRNA IL-15 DCs (line histogram), including the corresponding isotype control (grey filled line). IFN-γ, interferon gamma; mDC, mature dendritic cells.

comparable among untreated IL-15 DCs (163.4 ± 6.19 pg/mL), control luci siRNA IL-15 DCs (164.7 ± 18.52 pg/mL) and PD-L siRNA IL-15 DCs (152.8 ± 31.69 pg/mL, n = 6) in 24-hour washout supernatants (**Figure 2D**). Furthermore, granzyme B secretion in 48h washout supernatant was studied as it is a hallmark of IL-15 DCs. While untreated and luci siRNA treated IL-15 DCs secreted 110.05 ± 17.58 pg/mL and 88.97 ± 8.35 pg/mL of granzyme B, respectively, PD-L siRNA treated IL-15 DCs secreted 75.98 ± 9.69 pg/mL (n=3). This difference was not significant, demonstrating retainment of the prototypic DC function. In summary, these data demonstrate that PD-L siRNA/SAINT-RED lipofection at the monocyte level is highly reproducible and has no impact on the yield and the prototypic characteristics of mature IL-15 DCs.

In Situ PD-L Silencing Improves IL-15 DC-Mediated Primary Cell Allo-Stimulatory Capacity in the NK Cell Compartment, but Not Autologous PD-1$^+$ NK Cell Activity

Next, to investigate the impact of *in situ* PD-1 ligand disruption on the defining stimulatory function of DCs, allogeneic cell proliferation was assessed in a 5-day allo-MLR model using primary allo-PBL transfected with PD-1-encoding mRNA achieving overexpression of the inhibitory receptor. PD-1 expression was expressed on 85.25 ± 1.26% of viable PBL 2 hours after mRNA electroporation (ΔMFI of 1791.0 ± 165.7) and was significantly higher compared to its mock-transfected counterparts (ΔMFI 693.0 ± 102.0; **Figure 3A**, $p = 0.0058$, n=5). At the timepoint of analysis of supernatant and CFSE dilution, PD-1 expression after

TABLE 2 | Summary of yield, viability and post-cryopreservation (cryo) recovery of no siRNA IL-15 DCs (no), luciferase siRNA IL-15 DCs (luci) and PD-L siRNA IL-15 DCs (PD-L) cultured in 6-well plates or T75 flasks. Values are expressed as mean ± SEM.

		No	Luci	PD-L
Monocyte – IL-15 DCs yield	6-well plate	44.55 ± 3.75%	45.19 ± 2.42%	62.50 ± 2.42%
	T75	59.37 ± 1.81%	57.83 ± 1.85%	59.65 ± 1.73%
	All	58.02 ± 1.79%	56.68 ± 1.77%	59.90 ± 1.59%
Viability	6-well plate	94.38 ± 0.57%	86.83 ± 3.95%	91.68 ± 2.79%
	T75	93.59 ± 0.54%	92.51 ± 0.79%	93.64 ± 0.64%
	All	93.66 ± 0.49%	92.03 ± 0.81%	93.47 ± 0.62%
Post-cryo recovery		70.64 ± 5.58%	81.45 ± 7.69%	83.15 ± 7.07%

5 days of coculture with IL-15 DCs, increased to 15.61 ± 1.08% for mock-transfected PBL and decreased for PD-1-electroporated PBL to 22.80 ± 2.52%.

Anticipating increased inhibitory signaling, PD-1-overexpressing PBL (black bars) released lower amounts of IFN-γ than the untransfected PBL (grey bars) upon stimulation with any of the IL-15 DCs, which was only significant in luci siRNA IL-15 DC stimulated PBLs (p = 0.027; **Figure 3B**). As a positive control, stimulation with PHA/IL-2 resulted in the highest secretion of IFN-γ exceeding the detection ranges, confirming functionality of PBL engineered to overexpress PD-1. PD-1$^+$ PBL stimulated with control IL-15 DCs were capable of inducing IFN-γ (46.07 ± 20.38 pg/mL, no siRNA IL-15 DCs; 44.80 ± 27.06 pg/mL IFN-γ, luci siRNA IL-15 DCs), although not significantly higher than unstimulated PD-1$^+$ PBL (11.97 ± 0.72 pg/mL IFN-γ), demonstrating the presence of inhibitory activity by introduced PD-1. Disrupting PD-1 ligand-mediated signaling by the DCs, PD-L siRNA IL-15 DCs triggered higher amounts of IFN-γ in PD-1-overexpressing PBL (101.33 ± 33.09 pg/mL) although not significant (**Figure 3B**). Interestingly, IFN-γ production by non-electroporated PBL was significantly higher when stimulated by PD-L siRNA IL-15 DCs (171.14 ± 75.46 pg/mL) compared to IL-15 DCs without siRNA treatment (68.32 ± 23.00 pg/mL, p = 0.022, n=5), but not by luci siRNA IL-15 DCs (141.31 ± 50.90).

Demonstrating favorable IFN-γ secretion by primary PBL stimulated by PD-L siRNA IL-15 DCs, we evaluated potential preferential expansion of PBL subsets by differently treated IL-15 DCs. DCs were co-cultured with CFSE-stained PBL and CFSE dilution after 5 days served as a measure for cell proliferation (**Figures 3C, D**). While all IL-15 DCs possess allo-stimulatory capacity, no significant increase in proliferation could be measured in CD4$^+$ T cells and CD8$^+$ T cells. Interestingly, NK cells stimulated with PD-L siRNA IL-15 DCs proliferated 6.10 ± 1.26 times more than unstimulated NK cells compared to 4.38 ± 0.71 times after stimulation with untreated IL-15 DCs (p = 0.048). Furthermore, we measured CD25 expression and CD69 expression, two activation markers on PBL at 1-day intervals during the allo-MLR coculture, but could not detect any difference between no, luci or PD-L siRNA treated IL-15 DCs at any of the timepoints (data not shown).

To further investigate the role of NK cells as effector cells in the immune response induced by IL-15 DCs and the relevance of the PD-1 signaling axis, autologous NK cells were electroporated with *PD-1* mRNA prior to coculturing with IL-15 DCs. Mock-electroporated NK cells showed a basal expression of PD-1 with a ΔMFI of 2619 ± 320.5 (**Figure 3E**), which increased to 4551 ± 396.4 after electroporation with PD-1 mRNA (p = 0.0006). In an autologous setting however, NK cells did not produce more IFN-γ in 24 hour and 48 hour co-cultures with PD-L siRNA IL-15 DCs (**Figure 3F**).

PD-L1/2-Silenced IL-15 DCs Disrupt PD-1-Mediated Suppression of WT1-Specific T Cell Activity

Aiming to assess the added value of PD-L silencing in IL-15 DCs in an antigen-specific manner we previously developed a PD-1 overexpressing model T-cell line that can be readily transfected with antigen-specific TCR-encoding mRNAs of interest (20). In line with previous findings (20), maximal PD-1 surface expression (> 95%) was confirmed in these stably transduced PD-1$^+$ 2D3 cells (**Figure 4A**). $WT1_{37\text{-}45}$-specific *TCR* mRNA electroporation reproducibly resulted in a mean expression level of 87.72 ± 4.09% of TCRαβ$^+$ PD-1$^+$ 2D3 cells, 2 hours after transfection (**Figure 4A**). Next, we analyzed the expression of nuclear factor of activated T-cells (NFAT)-driven eGFP reporter gene by viable CD8$^+$ PD-1$^+$ 2D3 cells. Expression of eGFP, following co-culture with untreated, luci siRNA-treated and PD-L siRNA-treated IL-15 DCs that are exogenously pulsed with $WT1_{37\text{-}45}$ peptide or left unpulsed, serves as a measure for $WT1_{37\text{-}45}$-specific T cell activity (**Figure 4B**). WT1/PD-L1/PD-L2 triple-negative U266 cells were also exogenously pulsed with $WT1_{37\text{-}45}$ peptide or left unpulsed, and were used as control APCs in this WT1-specific PD-1$^+$ T-cell model assay (23). To determine whether reduced eGFP expression was caused by inhibitory signals mediated by PD-1/PD-L interaction, we included a condition in which $WT1_{37\text{-}45}$ peptide and neutralizing anti-PD-1 antibody nivolumab were added to the co-cultures. Stimulation with U266 without antigen resulted in a mean background activation of 2.67 ± 0.60% eGFP$^+$ PD-1$^+$ 2D3 cells (n = 5). In the presence of specific antigen, PD-L$^-$ U266 cells induced robust eGFP expression levels of 37.90 ± 5.82% and resulted in equally high T-cell activation when PD-1 was blocked by pre-incubation with the neutralizing antibody (40.02 ± 5.47% eGFP$^+$ PD-1$^+$ 2D3 cells), indicating that the maximal antigen-specific eGFP signal was reached (**Figure 4B**).

In line with control U266 cells, low background eGFP expressions were detected upon stimulation with untreated (no) IL-15 DCs (1.94 ± 0.32%), luci siRNA IL-15 DCs (2.03 ± 0.31%) and PD-L siRNA IL-15 DCs (2.21 ± 0.36%; for 7 independent donors) in the absence of peptide. Co-culture of TCR-engineered PD-1$^+$ 2D3 cells with non-silenced IL-15 DCs, which showed an average expression of 97.57 ± 2.13% PD-L1 and 9.80 ± 4.78% PD-L2 (7 independent donors) and that were pulsed with the corresponding $WT1_{37\text{-}45}$ peptide, resulted in low eGFP expression (14.24 ± 3.56%), similar to coculture with luci siRNA IL-15 DCs (13.64 ± 2.94% eGFP$^+$ PD-1$^+$ 2D3 cells). eGFP levels were not significantly different in these two conditions from the negative control without peptide. However, blocking of PD-1/PD-L interaction with nivolumab disrupted the suppressive effect, resulting in maximal eGFP expression by $WT1_{37\text{-}45}$-specific TCR-engineered PD-1$^+$ 2D3 cells when co-cultured with peptide-pulsed no siRNA IL-15 DCs (38.81 ± 4.13%) and luci siRNA IL-15 DCs (39.66 ± 3.08% eGFP$^+$ PD-1$^+$ 2D3 cells). These results confirm that the nearly absent T-cell activity is caused by active PD-1-mediated inhibition. Proving the concept of enhancing the antigen-specific T cell-stimulating capacity of IL-15 DCs by *in situ* silencing of PD-1 ligands, $WT1_{37\text{-}45}$ peptide-loaded PD-L-silenced IL-15 DCs – with mean relative expression of 16.58 ± 2.05% PD-L1 and 27.91 ± 7.70% PD-L2 – induced significantly higher specific T cell activation (26.04 ± 3.60% eGFP$^+$ PD-1$^+$ 2D3 cells) than their non-silenced counterparts (p = 0.035 compared to no siRNA IL-15 DCs and p = 0.024 compared to luci siRNA IL-15 DCs), approximating the

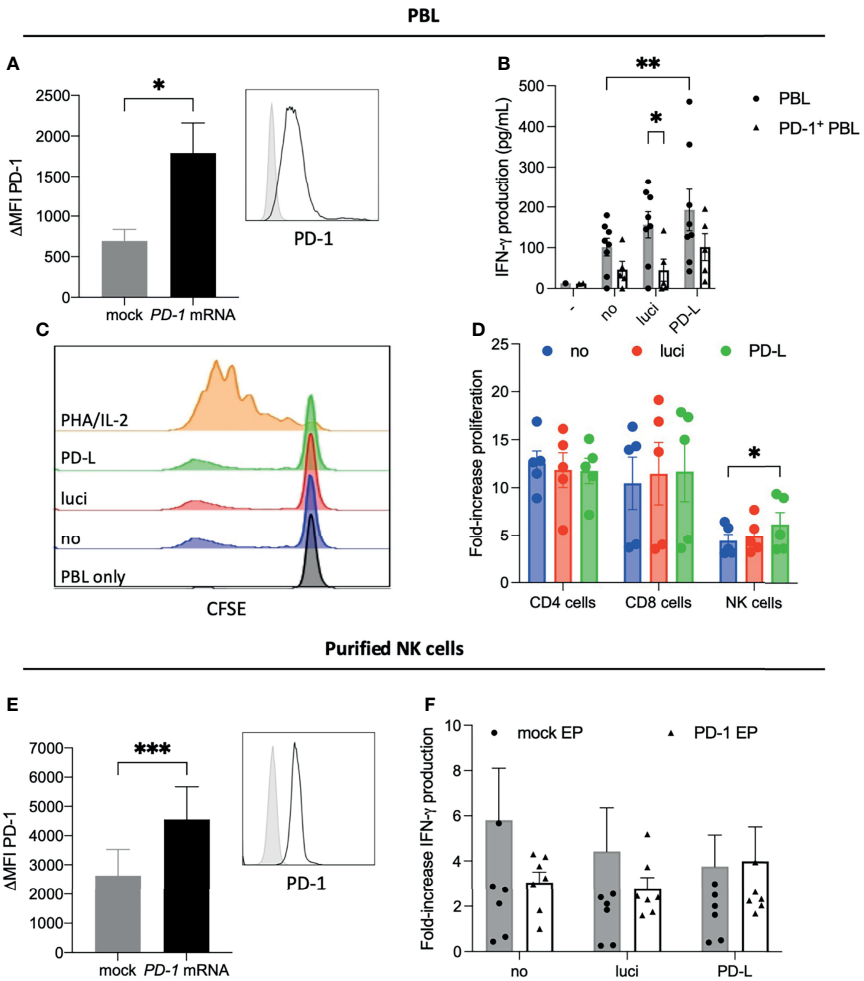

FIGURE 3 | Stimulatory effect of siRNA engineered IL-15 DCs on primary human effector cells. **(A)** Mean fluorescence intensity of PD-1 surface expression on viable PBLs 2 hours after mock and *PD-1* mRNA electroporation (n = 3). Inset represents histogram overlay of PD-1 expression over isotype control (filled histogram). **(B–D)** Allo-stimulatory capacity of untreated (no), control siRNA (luci) or PD-L siRNA IL-15 DCs in a 5-day MLR with PBL (mock; circle symbols) and *PD-1* mRNA-electroporated PBL (PD-1$^+$ PBL; triangle symbols). **(B)** Concentration IFN-γ in cell-free culture supernatant after 5 days stimulation (mean ± SEM; n = 9 in 4 independent experiments). **(C)** Degree of CFSE dilution in total viable PBL fraction is shown for one representative example of 6 donors, including a positive control with PHA/IL-2 and a negative control with unstimulated PBL. **(D)** Fold-increase of CFSE dilution upon stimulation with IL-15 DCs compared to their respective unstimulated mock-electroporated PBL, among singlet/Aqua live/dead$^-$ gated CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$ and CD3$^-$CD56$^+$ NK cells (mean +- SEM, n = 6 in 3 independent experiments). **(E)** PD-1 expression on purified mock-electroporated or *PD-1* mRNA-electroporated NK cells (n = 8). Histogram shows a representative example of PD-1 expression in mock EP NK cells (grey filled histogram) and PD-1 EP NK cells (black unfilled histogram). **(F)** Fold-increase in concentration IFN-γ in 48h supernatants of cocultures of purified mock or *PD-1* mRNA-electroporated NK cells with autologous no, luci, or PD-L siRNA IL-15 DCs (n = 8). For **(A, E)** an unpaired t-test was used upon confirming normal distribution and homoscedasticity. For **(B, D)** the non-parametric Friedman test was used, *p < 0.05, ***p < 0.001. CFSE, carboxyfluorescein succinimidyl ester; EP, electroporation; IFN-γ, interferon gamma; MFI, mean fluorescence intensity; NK cell, natural killer cell; PBL, peripheral blood lymphocytes; PD-1, programmed death-1. **, 0.01.

maximal activation potential in the presence of neutralizing anti-PD-1 antibody (38.67 ± 3.88% eGFP$^+$ PD-1$^+$ 2D3 cells).

We further exploited the possibilities of this PD-1$^+$ 2D3 based T-cell assay, by evaluating the tumor antigen-presenting capacity of PD-L-disrupted IL-15 DCs loaded with mRNA encoding the full-length WT1 protein. Contrary to exogenous peptide pulsing, transfection of DCs with *WT1* mRNA allows, in principle, endogenous processing and presentation of the full WT1 epitope repertoire. *WT1* mRNA transfection efficiency was demonstrated by intracellular WT1 protein expression with flow cytometry (**Figure 4C**). Mean expression levels of intracellular WT1 4 hours after *WT1* mRNA electroporation were comparable for all conditions, with 35.97 ± 11.14% WT1$^+$ U266 cells (n=4), 47.31 ± 10.23% WT1$^+$ siRNA untreated (no) IL-15 DCs, 53.27 ± 11.03% WT1$^+$ luci IL-15 DCs and 47.75 ± 12.51% WT1$^+$ PD-L siRNA IL-15 DCs (n=7; **Figure 4C**, representative example). To assess whether *WT1* mRNA-loaded and PD-L silenced IL-15 DCs can efficiently stimulate TCR-engineered PD-1$^+$ 2D3 cells in an antigen-specific manner, co-cultures of WT1$_{37\text{-}45}$ TCR-engineered PD-1 and the different IL-15 DCs conditions were set up similar to those used with WT1$_{37\text{-}45}$ peptide pulsing (**Figure 4D**). In line with non-peptide pulsed control U266 cells, mock-electroporated (no *WT1*

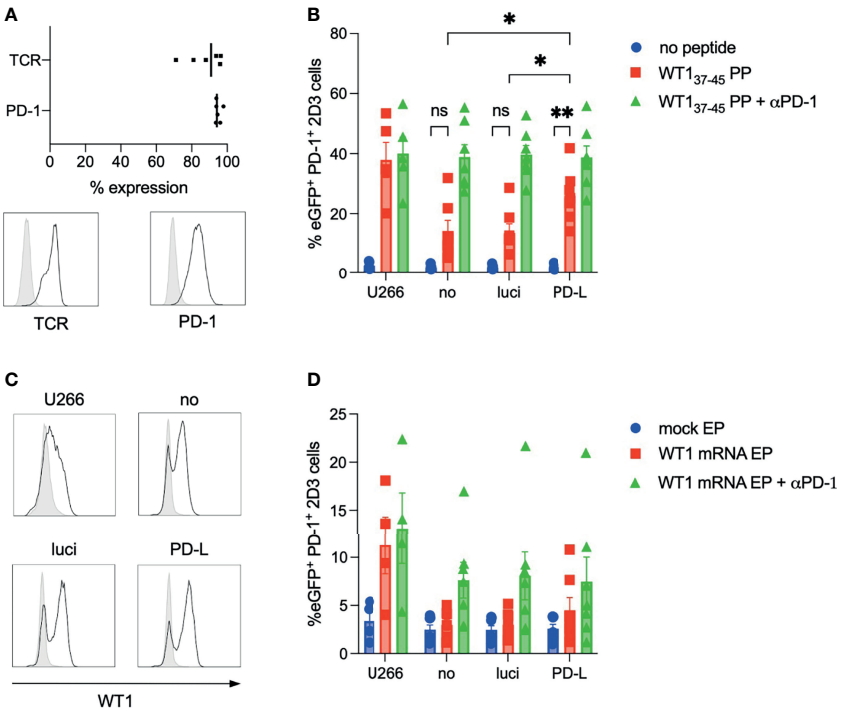

FIGURE 4 | Improved WT1-specific T cell activation by silencing of PD-1 ligands in a model PD-1$^+$ 2D3 T-cell model assay. **(A)** Percentage of PD-1 and TCRαβ surface expression in TCRαβ-deficient, PD-1-transduced and *WT1*$_{37\text{-}45}$ *TCR* mRNA-electroporated 2D3 cells (n = 6, upper panel) and representative histogram overlays (lower panel) of TCRαβ and PD-1 staining (black line) with their respective isotype (grey filled line). **(B)** Percentage of eGFP-expressing 7-AAD$^-$ CD8$^+$ PD1$^+$ 2D3 cells (n = 7) after overnight co-culture with untreated (no), control siRNA (luci) and PD-L siRNA (PD-L) IL-15 DCs that were either left unpulsed (no peptide) or pulsed with WT1$_{37\text{-}45}$ peptide (PP). In a third condition, IL-15 DCs were peptide pulsed and neutralizing anti-PD-1 antibody (nivolumab) was added to the co-culture (WT1$_{37\text{-}45}$ PP + αPD-1). **(C)** Representative histogram overlays of intracellular WT1 expression 4 hours after *WT1* mRNA electroporation (black line) versus mock (no mRNA) electroporation (grey filled line) of control APCs U266 cells and untreated (no), control siRNA (luci) and PD-L siRNA (PD-L) IL-15 DCs. **(D)** Percentage of eGFP-expressing 7-AAD$^-$ CD8$^+$ PD1$^+$ 2D3 cells (n = 7) after co-culture with mock-electroporated or *WT1* mRNA-electroporated PD-L$^-$ U266 cells or untreated (no), control siRNA (luci) or PD-L siRNA (PD-L) IL-15 DCs. Additionally, co-cultures with WT1 mRNA-electroporated APCs were pre-incubated with nivolumab. eGFP, enhanced green fluorescent protein; EP, electroporation; ns, not significant; PD-1, programmed death-1; TCR, T-cell receptor; WT1, Wilms' tumor protein-1. *, 0.05; **, 0.01; NS, not significant.

mRNA) U266 cells triggered low background percentages eGFP expression (3.37 ± 0.99% eGFP$^+$ PD-1$^+$ 2D3 cells). However, endogenously processing of WT1 in PD-L$^-$ U266 cells after *WT1* mRNA electroporation triggered a 3-fold increase in eGFP expression compared to the mock-electroporated control (11.28 ± 3.00% eGFP$^+$ PD-1$^+$ 2D3, 4 independent replicates), although this difference is not statistically significant. Elevated eGFP expression induced by *WT1* mRNA-electroporated U266 cells suggests that WT1$_{37\text{-}45}$ peptide is indeed processed and presented on the surface of U266 cells, as also reported in (23). As observed with WT1$_{37\text{-}45}$ peptide-pulsed U266 cells, blocking PD-1 inhibitory signal with anti-PD-1 antibody on the PD-1$^+$ 2D3 cells had no impact on eGFP expression (13.07 ± 3.72% eGFP$^+$ PD-1$^+$ 2D3), since U266 cells do not express PD-1 ligands. Background T-cell activation by any of the mock-electroporated IL-15 DCs conditions was reproducibly low with average percentages of eGFP$^+$ PD-1$^+$ 2D3 cells of 2.49 ± 0.47% for no siRNA, 2.46 ± 0.44% for luci siRNA and 2.58 ± 0.45% for PD-L siRNA conditions (n=7). WT1$_{37\text{-}45}$ epitope presentation by *WT1* mRNA-electroporated IL-15 DCs induced 2.98 ± 0.59% (no siRNA) and 3.01 ± 0.53% (luci siRNA) eGFP$^+$ PD-1$^+$ 2D3 cells (n=7), therefore, not surpassing background activation levels. However, by blocking PD-1 signaling with nivolumab, WT1$_{37\text{-}45}$ specific activation by these untreated (no siRNA) *WT1* mRNA-loaded IL-15 DCs (7.62 ± 1.86%) and control siRNA (luci) *WT1* mRNA-loaded IL-15 DCs (8.08 ± 2.49%), was robustly 2,5-fold higher than the respective mock conditions (n=7), albeit not statistically significant. On the contrary, *WT1* mRNA-electroporated PD-L siRNA IL-15 DCs were capable of triggering above-background eGFP expression levels in PD-1$^+$ 2D3 cells in the absence of nivolumab (4.51 ± 1.31% eGFP$^+$ PD-1$^+$ 2D3 cells), compared to mock-electroporated PD-L siRNA IL-15 DCs although not significant. In the presence of nivolumab, PD-L siRNA IL-15 DCs were able to stimulate even more PD-1$^+$ 2D3 cells (7.47 ± 2.56% eGFP$^+$ PD-1$^+$ 2D3 cells) to the same level as no siRNA or luci siRNA IL-15 DCs.

DISCUSSION

Although DC vaccination has proven to be effective in a number of cancer patients, the lack of durable and widespread responses

urges the improvement of their immunogenicity through further research. In this paper, we developed a new DC vaccine candidate and evaluated it for its immunostimulatory properties. The discovery of the role of PD-1 in dampening anti-tumor immunity and antibodies targeting this inhibitory immune checkpoint or its ligands have revolutionized the field of cancer immunotherapy (24). This finding has led to PD-1/PD-L1 blocking antibody therapy being among the most often applied and promising cancer treatments (25). Increasing insights in the effects of systemic PD-1 checkpoint blockade has brought forward that this type of therapy is generally well tolerated, but can often result in several immune-related adverse effects, some of which have a severe nature (26). To increase the safety potential of PD-1 checkpoint blockade we hypothesized that neutralizing PD-1 ligands in a DC vaccine could increase the strength of the vaccine, while avoiding severe adverse effects by omitting systemic anti-PD-1 or anti-PD-L therapy. Notably, during the priming phase of T cells, which determines the fate of naïve T cells, the balance between co-stimulation and co-inhibition plays a major role. By decreasing the expression of co-inhibitory molecules, the T-cell fate is more likely to shift towards activation and induction of immunological memory, which is highly favorable as many TAAs are in fact self-antigens and thereby poor inducers of T cell responses (27).

In conventional IL-4 DCs, silencing PD-1 ligands resulted in increased IFN-γ production by allogeneic and antigen-specific T cells, both *ex vivo* and *in vivo* (17, 18). Importantly, combination with IL-15 transpresentation further augmented these responses (19). Our lab has previously generated a novel DC vaccine with improved immunostimulatory properties compared to conventional IL-4 DCs, so called-IL-15 DCs (7). In this study, we evaluated whether PD-L silencing could also further improve the immunopotency of the IL-15 DCs. Incorporation of PD-L siRNAs in the IL-15 DC protocol required alterations in the silencing protocol that was previously used in IL-4 DCs. PD-L siRNA transfection in IL-4 DCs was done by harvesting immature IL-4 DCs at day 3 and subsequent transfection with siRNAs followed by an extra differentiation step of 4 days and a maturation step of 2 days (16–19). However, the 3-day culture protocol of IL-15 DCs makes this siRNA transfection strategy unsuitable. Furthermore, as PD-L1 and PD-L2 expression is upregulated during the differentiation and maturation from monocytes into IL-15 DCs, delivery of siRNAs at the beginning of the culture (i.e. before proteins are expressed by the cells) would be most favorable. Therefore, we opted for delivery of siRNAs at the monocyte stage of the culture. However, this resulted in low yield and viability, possibly due to the more fragile nature of monocytes compared to immature DCs. Upon further optimizations we established a protocol where PD-1 ligands were efficiently silenced below a relative threshold value of 50% PD-L expression. This cut-off value was according to the criteria of the ongoing clinical trial using PD-L silenced IL-4 DCs by Dr. Schaap (NCT02528682, clinicaltrials.gov). An absolute knock-out of PD-L1 and PD-L2 was not considered favorable. First, expression of co-inhibitory molecules on the DCs can protect them from cytotoxic T-cell-mediated killing, prolonging their persistence and function. Second, although PD-L2 is usually seen as a co-inhibitory molecule, it is also demonstrated to have costimulatory properties, possibly *via* its interaction with repulsive guidance molecule B (28–30). Finally, a certain degree of co-inhibition is still desirable as overstimulation of immune cells can result in activation-induced cell death (31).

The role of effector T cells in the anti-tumor immune response is long-known and established, as well as PD-1 interactions in this context. On the contrary, involvement of NK cells and their PD-1 expression status in the immune response is only substantiated during the last decades, as is the bidirectional crosstalk between DCs and NK cells (32–37). In this regard, a prominent role of IL-15 is described, either membrane bound (9) or transpresented *via* IL-15 receptor α (38). Recently, many reports document on the expression of PD-1 observed in NK cells in relation to their functional exhaustion, both in cancer patients (39–41) and in patients with chronic infections (42). Given the crosstalk between NK cells and DCs on the one hand, and expression of PD-1 on exhausted NK cells and PD-Ls on DCs, on the other hand, there might be a rationale for PD-L silencing on DCs to also reinvigorate NK-cell functions. To robustly guarantee PD-1 expression on PBLs, we used *PD-1* mRNA electroporation to allow for pairwise analyses between unmanipulated and PD-1$^+$ PBLs (43). In line with our hypothesis, we demonstrated that PD-L siRNA IL-15 DCs increase cytokine production in an allo-MLR reaction. Because this effect was also present in luci siRNA IL-15 DCs, albeit at a less pronounced level, the contribution of the transfection reagent could not be excluded. Indeed, it has been shown, although not for SAINT-RED, that certain transfection reagents can result in differential gene expression due to their foreign nature as seen by the cell (44). However, as the effect was variable and still modest, we believe that the main effect in these set of experiments is due to the introduced siRNA and not the transfection reagent, although a synergistic function cannot be excluded. To our surprise, the stimulatory effect of PD-L silenced DCs was more pronounced in allogeneic PBLs that did not overexpress PD-1 compared to *PD-1* mRNA transfected PBLs, indicating such high inhibitory activity of the overexpressed PD-1, that it could not be overcome by PD-L silenced DCs. Indeed, PD-1^{high} T cells have distinct gene expression profiles compared to PD-1^{low} and PD-1^{intermediate} T cells, with the first expressing high levels of genes related to exhaustion (45). Although we did not induce PD-1 expression in a physiological manner, thereby generating a *bona fide* exhaustion model, the mere introduction of PD-1 was sufficient to inhibit IFN- γ secretion by PD-1$^+$ PBLs. Attempting to ascribe this IFN- γ production to a certain cell type, a proliferation assay identified NK cells, but not T cells to be expanded after stimulation with PD-L siRNA IL-15 DCs. Further focusing on NK cells, no difference could be seen in IFN-γ production by NK cells between no siRNA IL-15 DCs and PD-L siRNA IL-15 DCs in an autologous setting. It is likely that absence of killer immunoglobulin-like receptor ligand mismatch in an autologous setting limits the IFN-γ production by NK cells (46). The absence of a significantly increased proliferative

response in the T-cell compartment by PD-L downregulated DCs is in accordance with previous findings by others (47). Because the true potential of DC vaccination on T-cell activation relies on their ability to stimulate antigen-specific T cells, we focused on the $WT1_{37\text{-}45}$ epitope for the remainder of the paper. In an in-house developed assay to assess the PD-1^+ T-cell stimulating capacity of APCs, PD-L silenced IL-15 DCs showed stronger T-cell stimulatory capacities compared to IL-15 DCs with naturally expressed PD-1 ligands. Robustly demonstrated when DCs were loaded with one specific epitope, PD-L silenced DCs also outperformed their control counterparts when loaded with WT1-encoding mRNA. The lower response in this setting can be explained by the fact that the 2D3 cells in this assay only recognize one specific epitope which is the exact epitope used in the peptide-pulsed conditions, while mRNA loading results in multi-epitope presentation. Towards clinical implementation, mRNA loading is of great interest as this thus can result in a multi-epitope immune response by both $CD4^+$ and $CD8^+$ T cells (48). In this way other cancer-related epitopes might be presented that are not yet known. Moreover, this strategy requires no prior knowledge about the HLA-haplotype of patients (49), while the peptide pulsing approach requires the expression of correct HLA molecules.

The data in this paper demonstrate that PD-L siRNA IL-15 DCs are capable of stimulating tumor-antigen T cells, even when PD-1 is overexpressed. In this way, PD-L siRNA IL-15 DCs might reinvigorate exhausted tumor-reactive $CD8^+$ T cells. In the context of tackling inhibitory mechanisms with DC vaccination, regulatory T cells (Tregs) are gaining interest regarding the immune suppressive tumor micro-environment and their significance as a therapeutic target in cancer (50–53). It has been reported that PD-L1-mediated interactions with naïve $CD4^+$ T cells play an important role in the development, maintenance and function of inducible Tregs (54). Using nanoparticles coated with or without PD-L1, Francisco and colleagues demonstrated that in the absence of PD-L1 the development of inducible Tregs was reduced (54). Thus, PD-L siRNA IL-15 DCs might also be beneficial in the generation of a less immune suppressive tumor micro-environment. Further research is warranted to fully delineate the added value of PD-L siRNA IL-15 DCs as a potential new anticancer vaccine.

CONCLUSION

The PD-1/PD-L checkpoint axis is an important mediator of exhaustion in several immune effector cells in cancer. To advance next-generation DC vaccines with increased immunopotency, we successfully developed a robust protocol incorporating disruption of PD-1 ligands in our latest short-term cultured IL-15 DC vaccine, preserving all prototypic DC phenotype and functional characteristics. Ultimately designed to induce a durable tumor-specific immune response, PD-L silenced IL-15 DCs were capable of rescuing antigen-specific cytotoxic T cells from PD-1-mediated inhibition. Further corroborating the superior potency of short-term IL-15 DCs, the combination of immune stimulatory components during DC differentiation and maturation with *in situ* checkpoint inhibition supports further clinical translation.

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 Comité voor Medische Ethiek, Universiteit Antwerpen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MV and EL conceptualized the research. MV, DF, HR, LB, and SP performed the experiments. MV and EL wrote the manuscript. MV, DF, DC-D, HR, LB, SP, WH, ES, and EL critically reviewed and edited the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.734256/full#supplementary-material

Supplementary Figure 1 | Schematic overview of the PD-L siRNA monocyte-derived 3-day IL-15 DC culture protocol. After a 1-hour incubation of monocytes with lipoplexes of SAINT-RED (10 µL) with a mixture of PD-L1:PD-L2 siRNAs (2 µg siRNAs; 2:1 ratio), cells were differentiated for 24 to 48 hours and matured overnight into PD-L siRNA IL-15 DCs, without intermediate harvesting. DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; hAB, human pooled serum; iDC, immature dendritic cell; IFN, interferon; IL, interleukin; PD-L,

programmed death-1 ligands; PD-L1, programmed death-1 ligand 1; PD-L2, programmed death-1 ligand 2; PGE, prostaglandin; siRNA, small interfering ribonucleic acid; TNF, tumor necrosis factor. **Supplementary Figure 1** was created with BioRender.

Supplementary Figure 2 | Gating strategy employed in the allo-MLR experiment. First cells were gated based on FSC/SSC and viability. Then $CD3^+$ cells were gated to select $CD4^+$ and $CD8^+$ cells downstream. $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were selected to study proliferation based on CFSE dilution. CFSE-dilution gate was set on unstimulated PBL.

Supplementary Figure 3 | Gating strategy used in 2D3 assay. First 2D3 cells were gated based on their scatter profile. Then viable cells were selected by means of 7-AAD. $CD8^+$ cells were then selected, depicting the $CD8^+$ 2D3 cells. Finally, T cell activation is measured on these cells by measuring eGFP expression. The $eGFP^+$ gate was set on unstimulated 2D3 cells.

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