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Supplementary Information for

NOD2 modulates immune tolerance via the GM-CSF-dependent generation of CD103^{+} Dendritic Cells

David Prescott, Charles Maisonneuve, Jitender Yadav, Stephen J. Rubino, Stephen E. Girardin and Dana J. Philpott

Dana J. Philpott
Email: dana.philpott@utoronto.ca

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Supplementary Methods

Mice

All animals were maintained under specific pathogen-free conditions at the University of Toronto Department of Comparative Medicine. In some cases, age- and sex-matched C57Bl/6J wild-type mice were purchased from Jackson Laboratories and used at 6-10 weeks of age. Otherwise, mice were obtained from their respective suppliers and bred in-house to control for littermate effects. *Nod2* (kind gift from Drs. Marco Giovannini and Jean-Pierre Hugot)*, Nod1* (Millenium Pharmaceuticals)*, Rip2* (kind gift from Dr. Richard Flavell)*, CSF2* (Jackson Labs)*, and Batf3* knockout mice (kind gift from Dr. Jennifer Gommerman) were maintained in a +/- by -/- breeding system. Tissue-specific knockout of *Nod2* was generated by crossing $Nod2^{fl/fl}$ mice (kind gift from Dr. Philip Rosenstiel) with *Itgax-Cre* (CD11c-Cre; dendritic cell specific; Jackson Labs) or *Lyz2-Cre* (Lysozyme M-Cre; myeloid cells; Jackson Labs), and maintaining these mice in a Cre+ by Cre- breeding system. All mouse experiments were conducted as approved by the University of Toronto Animal Care Committee in accordance with the regulations set by the Canadian Council of Animal Care.

Injections and Vaccinations

Muramyl Dipeptide (Invivogen), FK-156 (Fuijisawa, Inc. (Japan)), LPS-EB Ultrapure (Invivogen), Pam3CSK4 (Invivogen), recombinant Flagellin (Invivogen), Zymosan A from *Saccharomyces cervisiae* (Millipore Sigma), EndoFit Ovalbumin (Invivogen), Complete Freund's adjuvant (Millipore Sigma), and recombinant GM-CSF (Biolegend) were purchased from their respective suppliers and prepared at the doses indicated in the text in sterile PBS prior to being administered to mice via injection into the peritoneal cavity. Intrarectal administration of MDP and 50% ethanol was performed by anaesthetizing animals under gaseous isofluorane before inserting a plastic catheter 3cm into the anus and slowly injecting 100µl of fluid directly into the colon lumen. Mice were inverted during the recovery from anaesthetic to facilitate the retention of fluid in the colon lumen.

Tissue Collection

To prepare single cell suspensions of Splenocytes, MLN cells and thymocytes, organs were excised and infused with 500ul of 2mg/ml Collagenase D (Roche) and 0.5mg/ml DNAse I (Roche) in PBS prior to being minced into small pieces and incubated at 37°C for 20 minutes. This preparation was then passed through a 70µM nylon filter, and the remaining solid tissue mashed through the filter with the plunger of a 3ml syringe. For the spleen, red blood cells were then lysed using ammonium chloride lysis buffer (100mM NH_4CL, 10mM $KHCO_3$, 0.1mM Na_2EDTA). Peritoneal exudate cells were collected by injecting 3ml of ice-cold PBS containing 3% fetal bovine serum into the peritoneal cavity, briefly massaging the region, and collecting the resulting lavage in a 50ml tube. Lamina prorpria cells were collected by dividing colons into 1-2cm pieces, removing mucous with 5mM DTT in HBSS, removing epithelial cells with 5mM EDTA, then finally mincing the tissue into fine pieces and digesting with 0.2U/ml Liberase TM (Roche) and 200U/ml DNAse I (Roche) for 30 minutes. Digested pieces were then titruated through a 21g needle and filtered through a tube-top strainer to remove debris.

Flow Cytometry
Single cell suspensions were stained with LIVE/DEAD fixable aqua dead cell stain (ThermoFisher) to exclude dead cells and debris, as per the manufacturer's instructions. Suspensions were then incubated with Anti-Mo CD16/CD32 (ThermoFisher, Clone 93) in flow cytometry staining buffer (0.3%FBS, 0.1% NaAzide) to block non-specific binding of antibodies by Fc receptors, prior to staining for surface antigens with the antibodies listed in Table S1 (See *SI Appendix)*. For intracellular FOXP3 analysis, cells were surface stained as above prior to fixation and permeabilization of the cellular suspensions with eBioscience Foxp3/Transcription Factor Staining Kit (ThermoFisher) as per the manufacturer's instructions. Flow cytometric analysis was performed using the LSRFortessa Cell Analyzer (BD Biosciences) running FACSDiva acquisition software, and data analyzed using FlowJo v10.

Adoptive Transfer Experiments
Single-cell suspensions of purified OT1 T-cells and OT2 T-cells were produced by magnetic sorting of splenocytes collected from respective mice using the $CD8a^+$ or $CD4^+$ T Cell Isolation kits, mouse (Miltenyi Biotec). Recipient mice were injected with $5x10^5$ cells via the tail vein, and injected with 50μg of Endofit Ovalbumin ± MDP 24h later. Eight days later, mice were again injected with 50μg of Endofit Ovalbumin prior to tissue collection at day 11.

Bone Marrow Chimeras.
Bone marrow chimeras were created by lethally irradiating recipient mice with 2 rounds of 550cGy γ-irradiation (Gammacell-40, Nordion) separated by 4 hours. Bone marrow from sex-matched donors was collected from femurs and tibias, lysed of red blood cell content with ACK lysis buffer, and $4x10^6$ cells injected into recipient mice via the tail vein. Mice were maintained on 2g/L neomycin sulfate (Sigma-Aldrich) for 2 weeks, and rested for a subsequent 6 weeks to ensure the reconstitution of the hematopoietic compartment.

Quantitative RT-PCR
Sections of spleen and parietal peritoneum (≈$1cm^2$) or colon (3cm from the anus) were excised and homogenized in RLT lysis buffer (Qiagen) using a bead beater homogenizer. RNA was extracted from lysates using the RNeasy Mini Kit (Qiagen) per the manufacturer's instructions. Genomic DNA was removed from the sample using the Turbo DNAse-free kit (Invitrogen), and RNA reverse transcribed with the Superscript IV RT kit (Invitrogen). One μg of total cDNA was added in triplicate to each qPCR reaction containing PowerUP SYBR Green master mix (Applied Biosystems) and 250μM specific primers (See *SI Appendix* Table S2), and run on a CFX384 Touch Real-Time thermal cycler (Bio-Rad). Relative gene expression was determined by the delta-delta Ct method, with 18s rRNA used as the housekeeping control.

DC Sorting and Affymetrix Microarray
C57Bl/6 mice were injected subcutaneously with $5x10^6$ FLT3L-expressing B16 melanoma cells (generous gift from Dr. Philippe Poussier) in order to boost endogenous

levels of cDC1s (64). Five days later, spleens were isolated and single cell suspensions isolated as above. Dendritic cells were purified from this preparation by magnetic sorting using the Pan Dendritic Cell Isolation Kit (Miltenyi Biotec), and sorted into respective groups (see figure 6) via fluorescent sorting using a FACS Aria IIIu (BD Biosciences). RNA was isolated from each sample using the RNeasy Micro RNA extraction kit (Qiagen), and samples submitted to The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children (Toronto, Canada) for Microarray Analysis. Briefly, samples were assessed for RNA integrity with the 2100 Bioanalzyer (Agilent), and suitable samples prepared with the WT Pico kit (Affymetrix) and hybridized on GeneChip Mouse Gene 2.0 microarray chips (Affymetrix) according to the manufacturer's instructions. Microarray data was normalized with the Robust Multi Array (RMA) method, and differential gene expression analysis performed using the open-source software geWorkbench (retrieved from: http://wiki.c2b2.columbia.edu/workbench/index.php/Home).

Data were pre-processed by filtering out probesets that lacked Entrez gene IDs, and for probesets with expression levels lesser than 5.0. Significantly altered probesets were determined by ANOVA followed by a step-down Holm-Bonferroni procedure. Gene Set Enrichment Analysis was performed using open-source software obtained from the Broad institute (retrieved from: http://software.broadinstitute.org/gsea/index.jsp), while Cell-Type Enrichment Analysis was performed using Cten, a web-based analytical tool identifying enriched cell types from microarray data (retrieved from: http://www.influenza-x.org/~jshoemaker/cten/, see reference (65))

***In vivo* BrdU incorporation assay**

Mice were injected i.p. with 1μg of Bromo-deoxyuridine (BrdU) in 100μL of PBS ± MDP, and BrdU concentrations maintained by including 0.8mg/ml BrdU in the drinking water. 24h later, spleens were harvested and analyzed for single-cell BrdU incorporation by flow cytometry using the BrdU for Flow Cytometry Staining Kit (eBioscience).

Statistical Analysis and Data Presentation

Statistical analysis was performed using Prism 6 (Graphpad Software) and the tests applied are described in the figure legends. Heatmaps were created using Heatmapper, an open-source online data presentation tool (retrieved from http://www.heatmapper.ca/, see reference (66)). Bar charts were created with DataGraph (Visual Data Tools, Inc.).

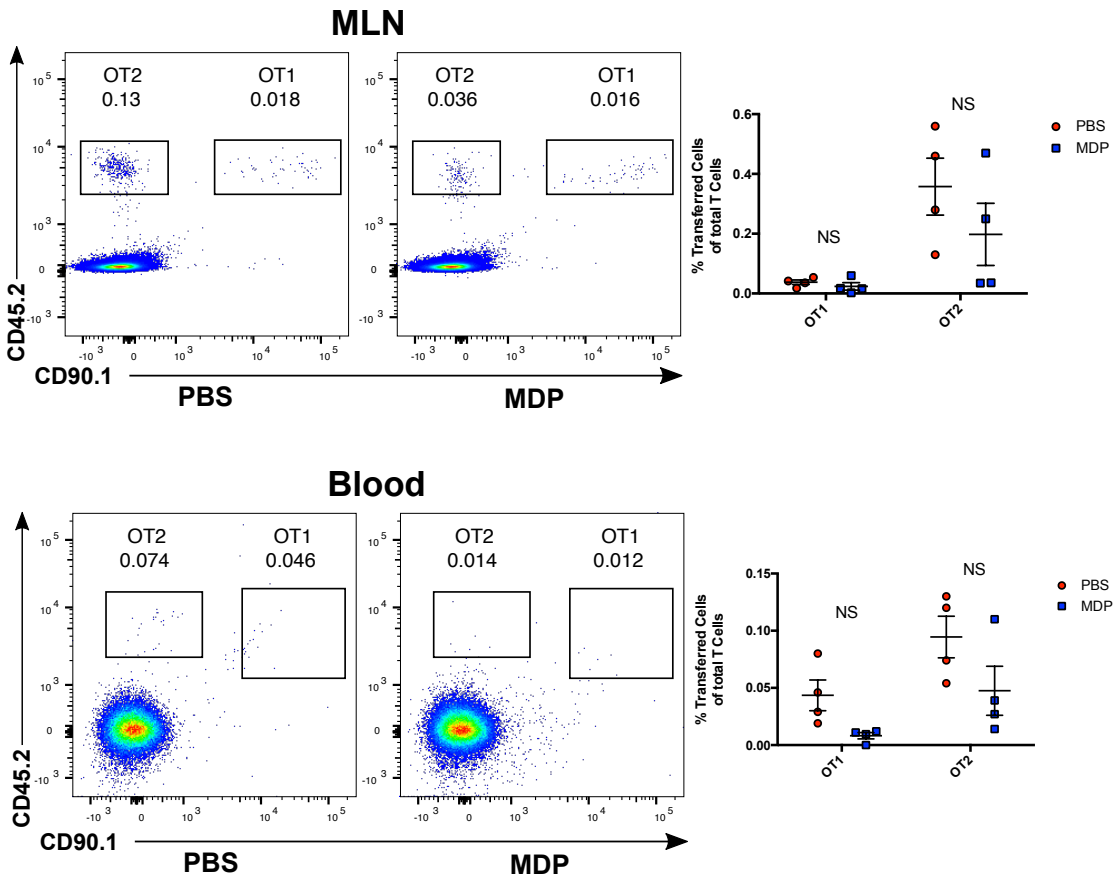


Fig. S1: MDP does not induce tissue sequestration of adoptively transferred transgenic T cells. Mice were adoptively transferred with OT1 ($CD45.2^+CD90.1^+$) and OT2 ($CD45.2^+$) cells and treated as in Figure 1d. Proportions of transferred cells in the MLN and Blood of individual CD45.1 recipient mice 3-days following re-challenge with ultrapure ovalbumin as assessed by flow cytometry from 1 representative experiment are shown. NS signifies no statistical significance between groups.

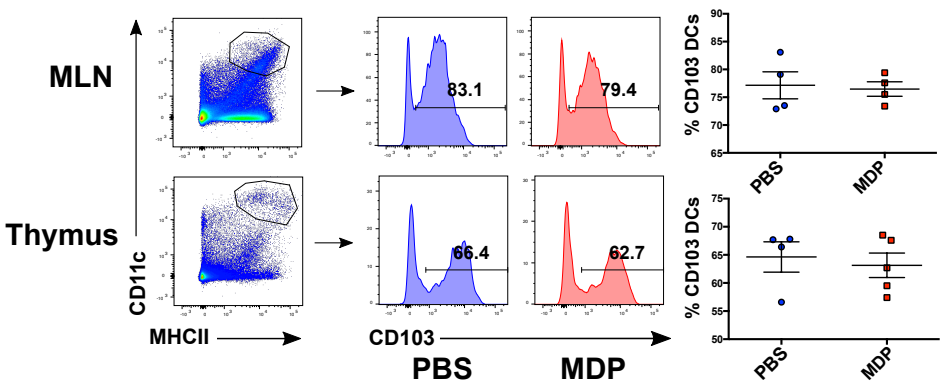


Fig. S2: Systemic administration of MDP does not alter CD103 expression on MLN or Thymic DCs Flow cytometry analysis of CD103 expression on $CD11c^{+}MHCII^{+}$ cells from murine mesenteric lymph nodes and thymus following injection with MDP (50µg i.p.).

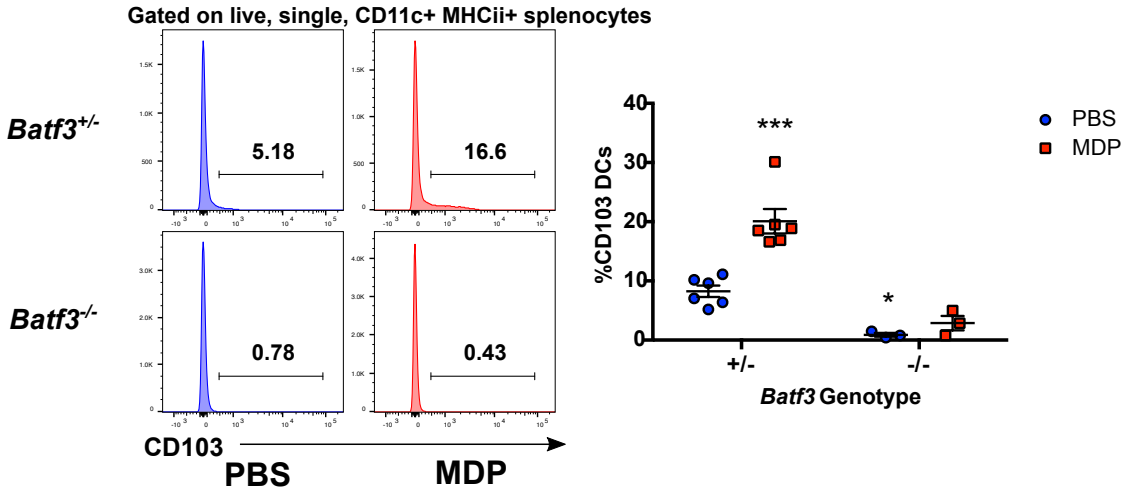


Fig. S3: MDP-induced increases in CD103 expression occur on BATF3-dependent cDC1 cells. CD103 expression on splenic dendritic cells following injection of MDP in mice lacking the transcription factor Batf3 (*Batf3*$^{-/-}$) or their heterozygote littermates (*Batf3*$^{+/-}$). Individual mice from two representative litters are denoted on the plots. Horizontal bars denote the mean, and error bars denote SEM. Asterisks denote significantly different from PBS-treated *Batf3*$^{+/-}$ mice as calculated by two-way ANOVA followed by Tukey's multiple comparisons test; * = $p<0.05$; *** = $p\leq0.001$.

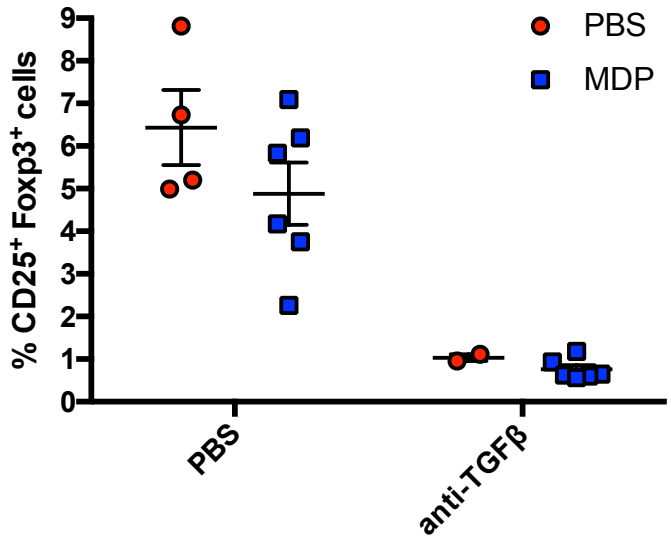


Fig. S4: Purified splenic CD205^{+}CD8α^{+} DCs spontaneously generate FoxP3^{+}CD25^{+} Tregs from naïve T cells when presenting cognate antigen. CD103^{low} cDC1 cells were FACS-sorted from the spleens of PBS-injected mice and CD103^{high} cells were sorted from the spleens MDP-injected mice (20,000 cells/well) and loaded with .03µg/ml OVA peptide (323-336). These cells were then co-cultured with MACS-purified naïve CD4 T cells collected from the spleens of OT2 mice (20,000 cells/well) in the presence or absence of 10µg/ml TGF-β neutralizing antibody for 5 days. CD4 T cells were then analyzed for FoxP3 and CD25 expression by flow cytometry to confirm the induction of Tregs.

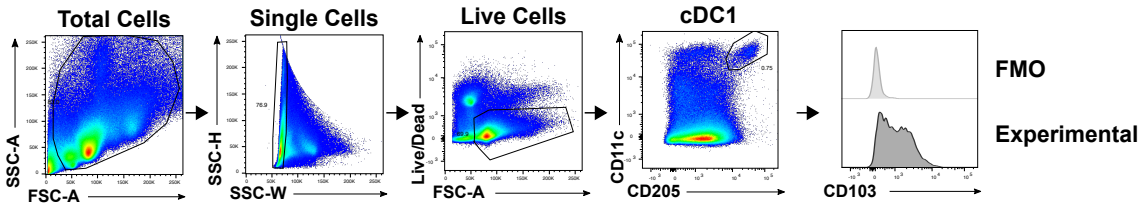


Fig. S5: Gating strategy employed to identify CD103+ cDC1s. For flow cytometry analysis, cDC1s are defined as live, single, $CD205^{+}$ $CD11c^{+}$ cells as shown in this example gating strategy. Further analysis identifies surface marker positive cells by setting gate borders using fluorescence minus one controls (FMO).

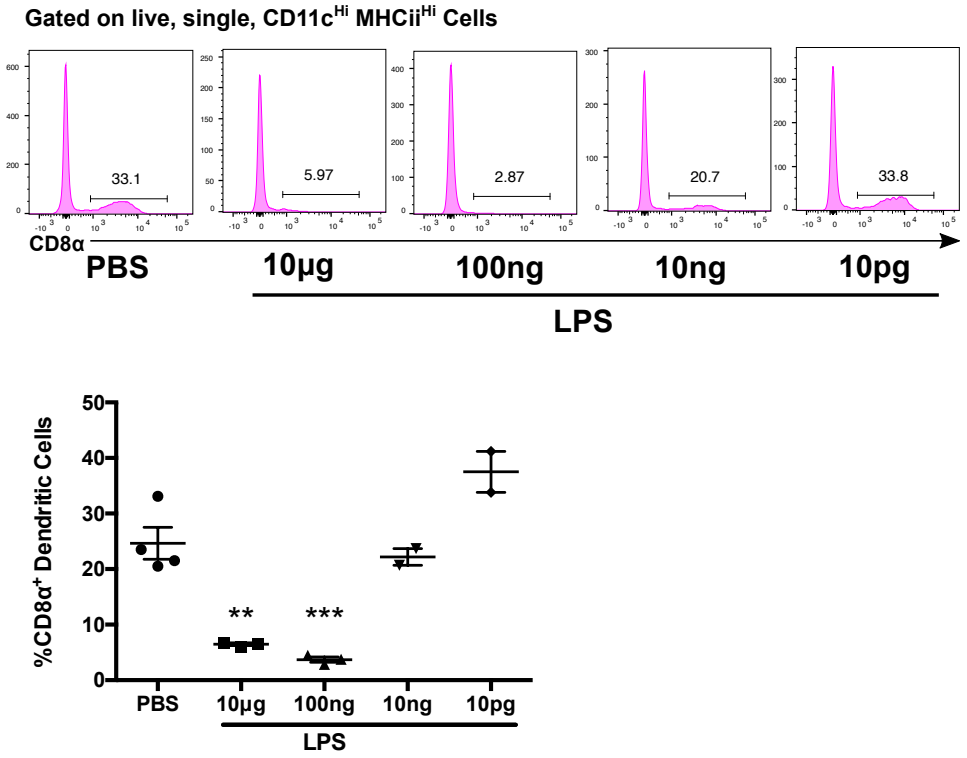


Fig. S6: LPS induces a dose-dependent disappearance of CD8α-expressing DCs. C57Bl/6 mice were injected with doses of LPS ranging from 10µg to 10pg, and splenocytes harvested 16h later and assessed by flow cytometry. Histograms show the proportion of dendritic cells ($CD11c^{hi}MHCii^{hi}$) that express CD8α at each dose of LPS. Data below show individual mice from one representative experiment. Horizontal bars denote the mean, and error bars denote SEM. Asterisks denote significantly different from PBS as calculated one-way ANOVA followed by Tukey's multiple comparisons test; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

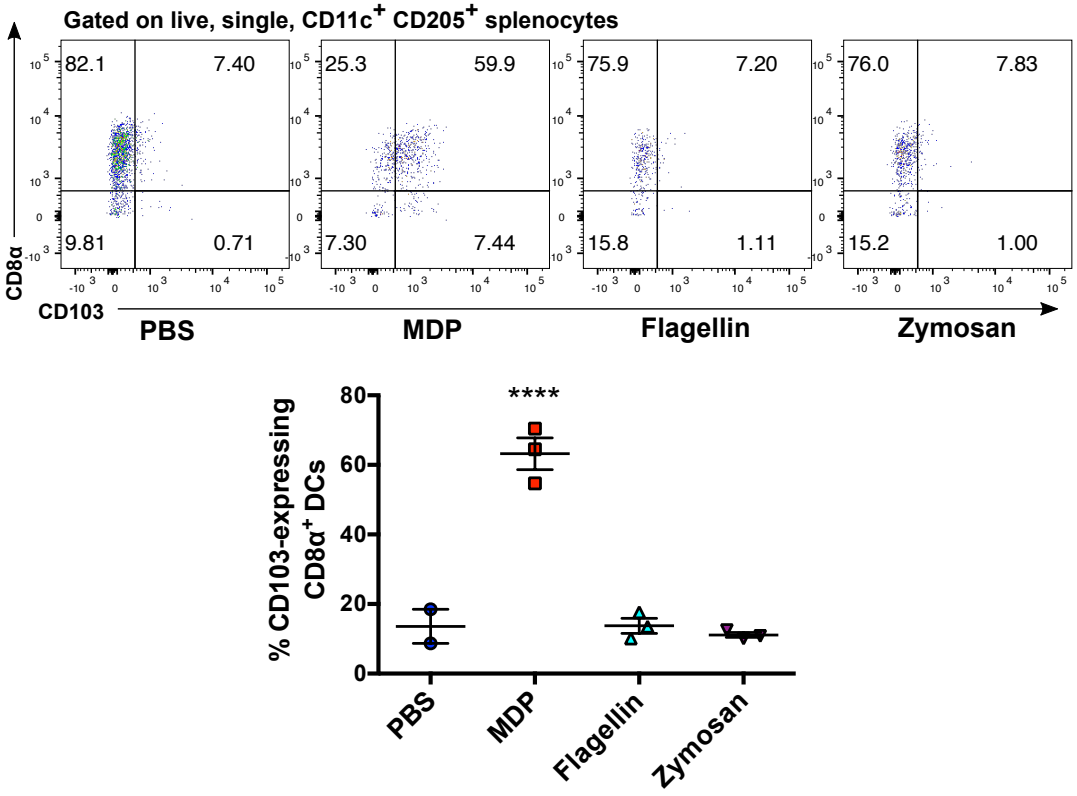


Fig. S7: Additional TLR ligands do not upregulate CD103 on splenic cDC1s. C57Bl/6 mice were injected i.p. with MDP (50µg), the TLR5 Ligand Flagellin (20µg), or the TLR2/6 ligand zymosan (10µg) or PBS vehicle control, and CD103 expression on splenic cDC1s assessed 16h later. DC subset marker and CD103 gate borders were drawn using fluorescence minus one (FMO) controls. Individual mice from one representative experiment are shown on each graph. Horizontal bars denote the mean, and error bars denote SEM. Asterisks denote significantly different from PBS as calculated by Student's T-test; ***** = P≤0.0001

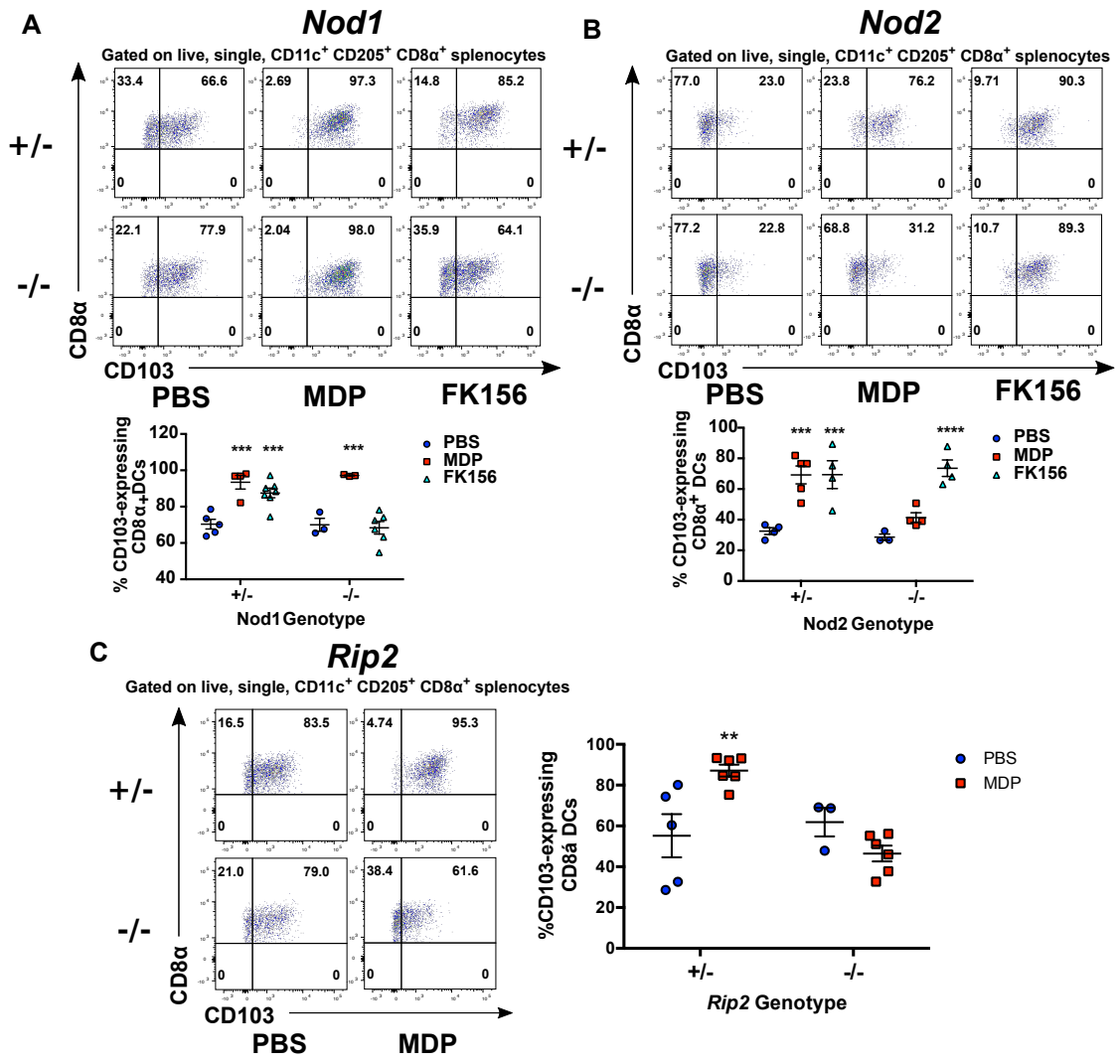


Fig. S8: FK156 and MDP induce CD103 specifically via activation of NOD1 and NOD2 in a RIP2-dependent manner. Flow cytometry analysis of CD103 expression by splenic CD8α$^+$ cDC1s collected from mice deficient for (A) *Nod1* (*Nod1*$^{-/-}$) or (B) *Nod2* (*Nod2*$^{-/-}$), along with their littermate heterzyogous controls (*Nod1*$^{+/-}$ or *Nod2*$^{+/-}$) following injection with MDP (50µg) or FK156 (50µg). Individual mice from 3 pooled litters are shown on each graph. (C) Flow cytometry analysis of CD103 expression on splenic CD8α$^+$ cDC1s collected from mice deficient in *Rip2* (*Rip2*$^{-/-}$) or their heterozygote littermates (*Rip2*$^{+/-}$) following injection with MDP (50µg). Individual mice from 2 pooled litters are shown on the graph. Horizontal bars denote the mean, and error bars denote SEM. Asterisks denote significantly different from PBS-treated heterozygote littermates as determined by one-way ANOVA followed by Tukey's multiple comparisons test; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

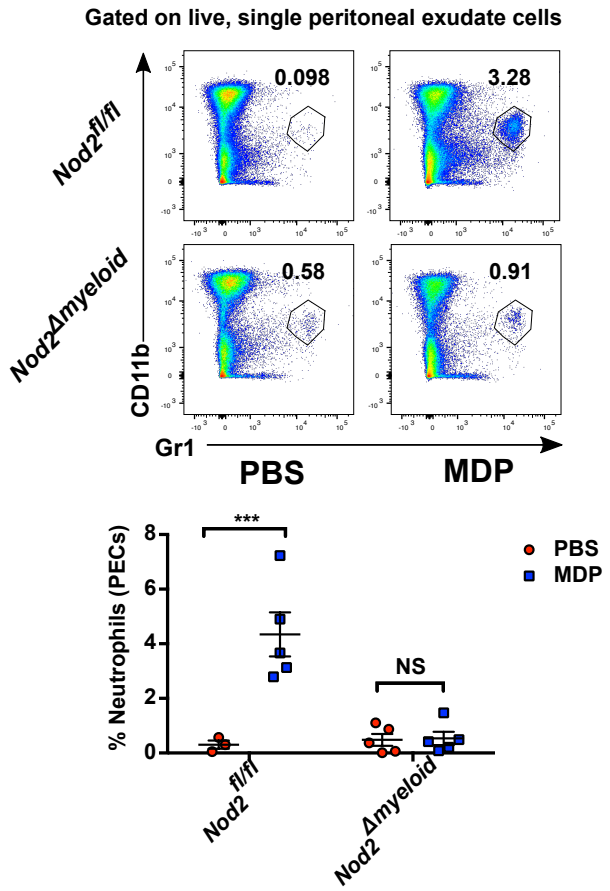
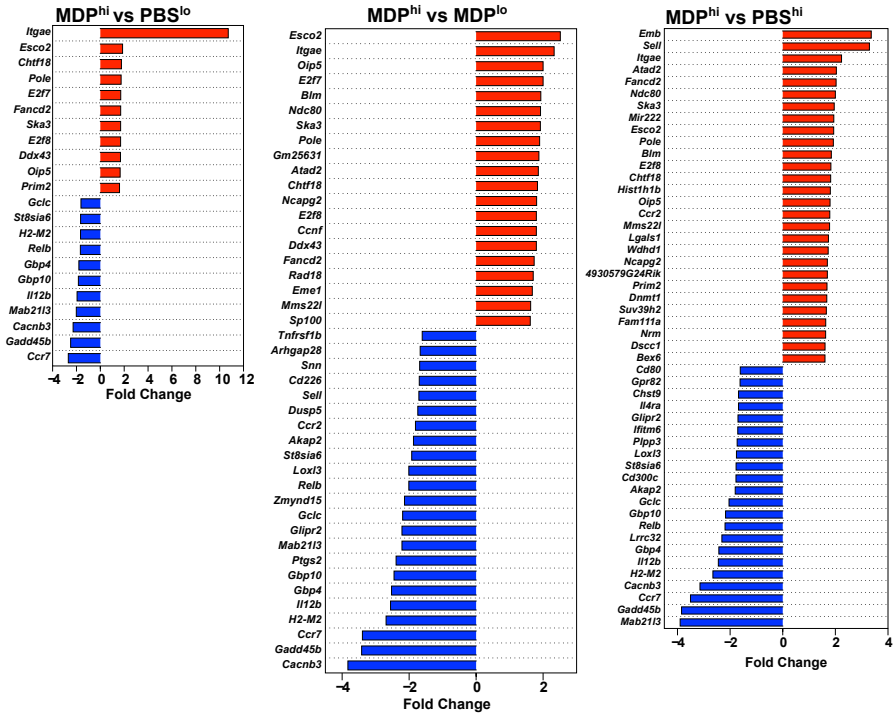


Fig S9. Flow cytometry analysis determining the proportion of neutrophils ($CD11b^{mid}Gr1^{hi}$) found in peritoneal lavages collected from *Nod2*$^{\Delta myeloid}$ and their *Nod2*$^{fl/fl}$ littermates 16h post-injection with MDP (50µg). Individual mice from two pooled litters are shown on the graph. Horizontal bars denote the mean, and error bars denote SEM. Asterisks denote significantly different from *Nod2*$^{fl/fl}$ littermate-matched PBS as determined by two-way ANOVA followed by Tukey's multiple comparisons test; *** = $p \leq 0.001$. NS denotes no statistical significance between linked groups.

a


MDP^hi vs PBS^lo
Itgae
Esco2
Chtf18
Pole
E2f7
Fancd2
Ska3
E2f8
Ddx43
Oip5
Prim2
Gclc
St8sia6
H2-M2
Relb
Gbp4
Gbp10
Il12b
Mab21l3
Cacnb3
Gadd45b
Ccr7
−4 −2 0 2 4 6 8 10 12
Fold Change
MDP^hi vs MDP^lo
Esco2
Itgae
Oip5
E2f7
Blm
Ndc80
Ska3
Pole
Gm25631
Atad2
Chtf18
Ncapg2
E2f8
Ccnf
Ddx43
Fancd2
Rad18
Eme1
Mms22l
Sp100
Tnfrsf1b
Arhgap28
Snn
Cd226
Sell
Dusp5
Ccr2
Akap2
St8sia6
Loxl3
Relb
Zmynd15
Gclc
Glipr2
Mab21l3
Ptgs2
Gbp10
Gbp4
Il12b
H2-M2
Ccr7
Gadd45b
Cacnb3
−4 −2 0 2
Fold Change
MDP^hi vs PBS^hi
Emb
Sell
Itgae
Atad2
Fancd2
Ndc80
Ska3
Mir222
Esco2
Pole
Blm
E2f8
Chtf18
Hist1h1b
Oip5
Ccr2
Mms22l
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Cd80
Gpr82
Chst9
Il4ra
Glipr2
Ifitm6
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Loxl3
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Cd300c
Akap2
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Relb
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−4 −2 0 2 4
Fold Change

b

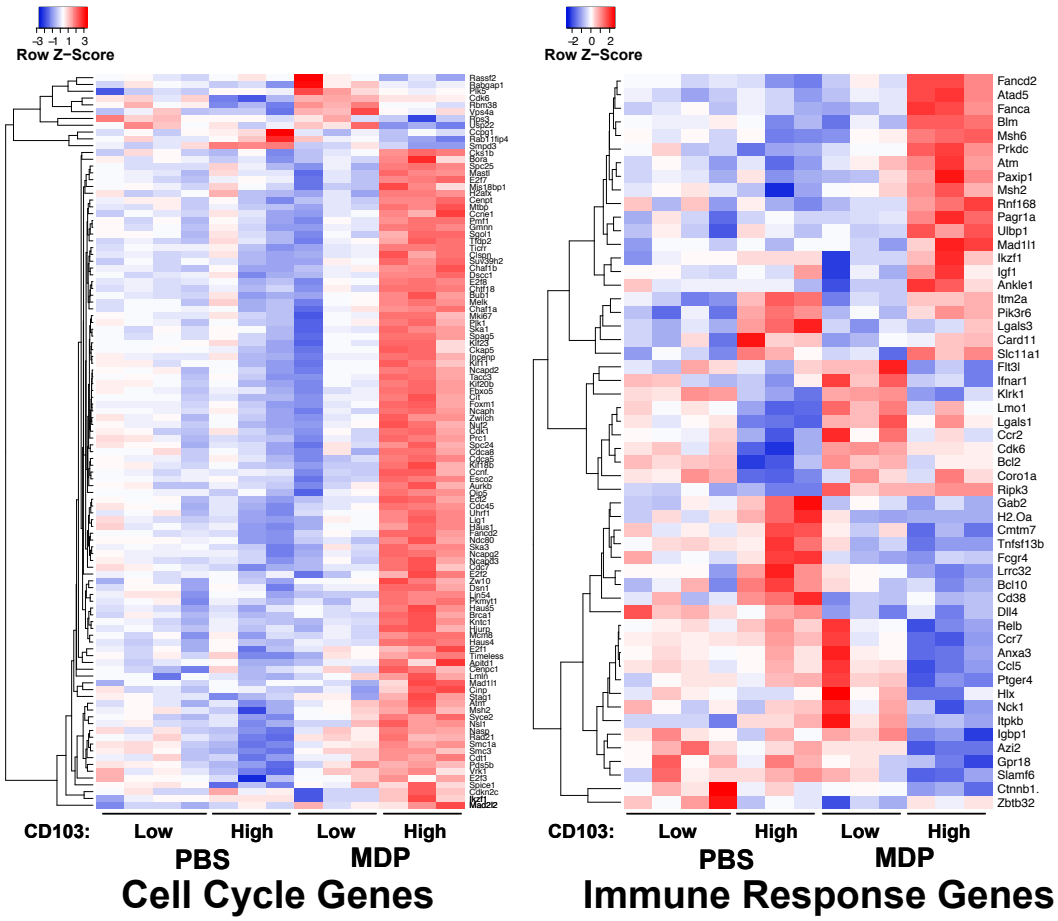

Figure S10: Significant changes in gene expression in CD103^{hi} cDC1s induced by MDP injection. Splenocytes from mice injected with MDP or PBS were sorted into CD103^{hi} and CD103^{lo} via FACS. Microarray analysis revealed a number of genes whose expression was significantly altered in MDP^{hi} dendritic cells. (a) Those genes that exhibited a fold-change of > 1.6 in comparison to the PBS^{lo}, MDP^{lo}, and PBS^{hi} DCs are listed here, where the size of the bars indicates the magnitude of the change, and the color of the bars indicating increases (red) or decreases (blue) in gene expression. (b) Heat map displaying genes described as being involved in the regulation of the cell cycle. Red indicates higher expression relative to the group average, while blue indicates decreased expression relative to the group average.

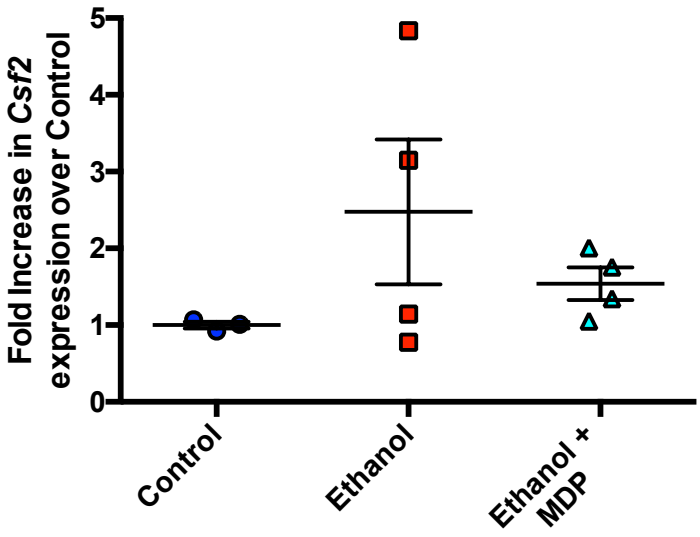

Figure S11: Intrarectal administration of ethanol or MDP induces *Csf2* gene expression in the colon. Gene expression analysis of whole-thickness colon tissue collected 2h post-administration of either PBS (control), 50% ethanol, or MDP delivered in 50% ethanol as assessed by qPCR. Fold expression over the average *Csf2* expression level in control mice was calculated using the ΔΔCT method. Individual mice from one experiment are shown on the graph.

Table S1: Antibodies Used in this study

TARGET	FLUOROPHORE	CLONE
CD4	FITC Or APC	RM4-5
FoxP3	APC	FJK-16S
TCRb	APC-eFluor 780	H57-597
CD45.2	PerCP-Cyanine 5.5	104
CD90.1	Super Bright 645	HIS51
CD11c	PE-Cyanine 7	N418
MHC Class II (I-A/I-E)	eFluor 450 or eVolve 655	M5/114.15.2
CD103	APC	2E7
CD8α	Alexa Fluor 700	53-6.7
CD205	PE-eFluor 610	205yekta
CD24	eFluor 450	M1/69
XCR1*	FITC	ZET
Clec9a	PerCP-eFluor 710	42D2
CD11b	APC-eFluor 780	M1/70
SIRPα	PE	P84
Gr1	Alexa Fluor 700	RB6-8C5
KLRG1	FITC	2F1
CD127	PE-Cyanine 7	SB/199
BrdU	FITC	BU20A

All antibodies purchased from Life Technologies with the exception of * (Biolegend)

Table S2: Sequences of PCR primers used in this study

Target	Forward	Reverse
CSF2	GGCTAAGGTCCTGAGGAGGAT	ACCTCTTCATTCAACGTGACAGG
18s	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC

References

64. Mach N, et al. (2000) Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* 60(12):3239–3246.

65. Shoemaker JE, et al. (2012) CTen: a web-based platform for identifying enriched cell types from heterogeneous microarray data. *BMC Genomics* 13(1):460.

66. Babicki S, et al. (2016) Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res* 44(W1):W147–53.