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MHC class II-restricted antigen presentation by plasmacytoid dendritic cells drives pro-atherogenic T cell immunity

Supplemental Material

Supplemental Methods

Mice

All experiments were approved by the Home Office, UK and were performed under PPL 80/2426. *Ldlr*$^{-/-}$ and µMT mice were purchased originally from Jackson labs and were on a C57Bl/6 background. *CD11c*-Cre x *Tcf4*$^{-/flox}$ and *CD11c*-Cre x *Tcf4*$^{+/flox}$ control littermates were originally generated in the lab of Prof. B. Reizis [1,2]. Mice lacking the pIII and pIV promoter elements of Class II transactivator (CIITA) (*pIII+IV*$^{-/-}$) [3,4] were maintained on a µMT (B cell deficient) background and µMT mice were used as controls. MHCII-deficient bone marrow was kindly provided by the lab of Sebastian Amigorena (INSERM U932, Institut Curie, Paris, France). For atherosclerosis experiments, male 6-8 week old *Ldlr*$^{-/-}$ mice were lethally irradiated (9.5 Gy) then injected *i.v.* with 1x10^7 bone marrow cells from donor mice. After 4 weeks recovery, mice were fed a high fat diet (21% Fat, 0.15% Cholesterol) for 6 or 8 weeks. In order to assess the effects of selective deficiency of *pIII+IV* on pDCs but not B cells, lethally irradiated *Ldlr*$^{-/-}$ mice were reconstituted with a mixture of 80% bone marrow from either µMT:*pIII+IV*$^{-/-}$ or control µMT mice and 20% bone marrow from WT C57BL6 mice. In this case, all B cells derive from the WT cells whereas 80% of all other leukocytes derive from µMT or µMT:*pIII+IV*$^{-/-}$ cells. In some experiments, 100 µg of anti-CD4 depleting antibody (clone YTS 191.1) [5] was injected every 10 days starting coincident with the start of HFD feeding.

Cell preparation from aorta

Cell suspension from aorta was prepared by enzyme digestion as previously described [6,7]. Briefly, *Ldlr*$^{-/-}$ mice were placed under terminal anesthesia and perfused with 2 mmol/L EDTA (Sigma-Aldrich, Gillingham, UK) in PBS via cardiac puncture to remove blood contamination from vascular tissue. After removal of aortas, a single cell suspension was obtained by incubation of aortic segments in an enzymatic suspension containing 450 U/mL collagenase type I, 125 U/mL collagenase type XI, 60 U/mL hyaluronidase, and 60 U/mL DNase (all from Sigma-Aldrich) in PBS containing 20 mmol/L Hepes at 37°C for 1 hour. Digested aortas were then mechanically disrupted through a 40-µmol/L cell strainer to release a single cell suspension. All the Abs used

for flow cytometry analysis of mouse aorta had been validated on cell suspensions from spleen/lymph nodes untreated or treated with the enzyme digestion cocktail.

Flow cytometry

Single cell suspensions of bone marrow, spleen, lymph node, blood and aorta were stained with fluorophore-conjugated antibodies (Supplemental Table 1) and analyzed using LSRII Fortessa (BD) or CyAN ADP (Beckman Coulter) flow cytometers. For intracellular staining, cells were activated with leukocyte activation cocktail (BD) for 4 h. Cells were fixed with IC fixation buffer (eBioscience) before intracellular staining. Cells were processed with Foxp3 buffer set (eBioscience) before staining with Foxp3. Data was analysed using FlowJo software (TreeStar, OR, USA). Dead cells were excluded based on FSc, SSc and positive staining for Live/Dead Aqua (Life Technologies). pDCs were defined as $CD11c^{lo}$ $PDCA1^{hi}$ $B220^{+}$ $CD11b^{-}$, cDC as $CD11c^{hi}$ $MHCII^{+}$, B cells as $B220^{+}$ IgM^{+} or $CD19^{+}$ lymphocytes, T cells as $CD4^{+}$ or $CD8^{+}$ lymphocytes, Treg as $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ $GITR^{+}$, monocytes as $CD11b^{+}$ $Ly6G^{-}$ and Ly6C low, intermediate or high, neutrophils as $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{+}$. Representative plots are shown in Supplemental Figure 1. In some experiments, Siglec-H (Miltenyi) was used as an additional pDC marker.

Analysis of *in vivo* antigen uptake/processing

To study the ability of aortic pDCs to present systemic antigen, we used the Eα-GFP/Y-Ae system as described previously [7,8,9]. Briefly, $Ldlr^{-/-}$ mice fed chow or HFD were i.v. injected either with 1 mg of Eα antigen or PBS, and were killed 4 hours later for FACS analysis. The APCs take up the Eα antigen, and the Eα peptide in the context of MHC ($I\text{-}A^{b}$) can be recognized by the Y-Ae mAb.

To determine the anatomical location of the antigen processing pDCs, $Ldlr^{-/-}$ mice fed HFD for 28 days were injected i.v. with 2.5 mg of DQ ovalbumin (DQ-OVA; Molecular Probes). After 1 h the aortic sinus was harvested, embedded in Tissue-Tec OCT (Tissue Tek, Sakura Finetek Europe, Zoeterwoude, the Netherlands) and snap frozen for immunohistochemical analysis. For staining, sections were fixed in acetone for 10 mins, air dried, and rehydrated with PBS before incubation in serum-free Protein Block (DakoCytomation) for 30 mins. pDCs were detected by staining for Siglec-H (440c, HyCult Biotech, Uden, The Netherlands). The primary antibody was detected using a Texas Red conjugated donkey anti-rat IgG (Jackson ImmunoResearch). Images

were taken by a Leica DFC340 FX video-camera (Leica Microsystems) connected to a fluorescence microscope (Leica DMRB) using the LAS software (vers. 2.8.1, Leica). Dapi was used to identify nuclei.

For *in vivo* OT-II stimulation, C57BL6 mice (4/group) were injected with 5 x 10^6 CFSE (Life Technologies)–labeled OT-II T cells. After 24h mice were injected into the left footpad with 1 x 10^5 μMT or μMT:*pIII+IV*$^{-/-}$ pDCs pre-incubated with ovalbumin (100 μg/ml) for 3h. OT-II T cell proliferation (CFSE dilution) was assessed after 3 days by flow cytometry in the popliteal lymph node, using spleen and the contralateral popliteal lymph node as internal controls.

***In vitro* dendritic cell culture**

Bone marrow pDCs and spleen CD11c$^+$ cells were isolated by negative and positive magnetic selection, respectively, according to the manufacturer's instructions using an AutoMACS Pro separator (Miltenyi). For cytokine production, purified pDCs (2 x 10^4) were treated with type A or B CpG or control GpC oligonucleotides (10 μg/ml; Invivogen). IFNα levels in supernatants were quantified by ELISA (eBioscience).

Antigen-specific T cell stimulation in vitro

OT-II CD4$^+$ T cells were incubated with cDCs or pDCs preincubated with 100 μg/ml ovalbumin (Sigma) and proliferation quantified after 3 days by ^{3}H thymidine incorporation over the final 18h. An I-A^b restricted murine T cell hybridoma (48-5T) recognising human ApoB100 [10] was added to pDCs or CD11c$^+$ cells preincubated for 4h with native human LDL (50 μg/ml; Intracel) in 0.5% serum-DMEM and incubated overnight. Supernatants were analysed for IL-2 levels by ELISA (Peprotech).

Analysis of atherosclerotic lesions

Total plasma cholesterol was quantified using a Cholesterol RTU kit (Biomerieux).

Aortic root atherosclerotic lesions were analysed by Oil Red O and CD3 staining as previously described [11]. Images were captured and analysed using a Leica DM6000B microscope and accompanying software.

Statistics

Results were presented as mean ± S.E. They were analyzed in GraphPad Prism (La Jolla, CA, USA) using unpaired t-test, non-parametric Mann Whitney U test, one way analysis of variance or two-way analysis of variance, as appropriate. A P value (two-sided) of <0.05 was considered significant. For analysis of

atherosclerotic lesions in the aortic root, the entire 10-section profile was analyzed by repeated measures two-way analysis of variance and the p value for between groups displayed.

Supplemental methods references

1. Ghosh, H. S., Cisse, B., Bunin, A., Lewis, K. L. & Reizis, B. Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. *Immunity* 33, 905–916 (2010).
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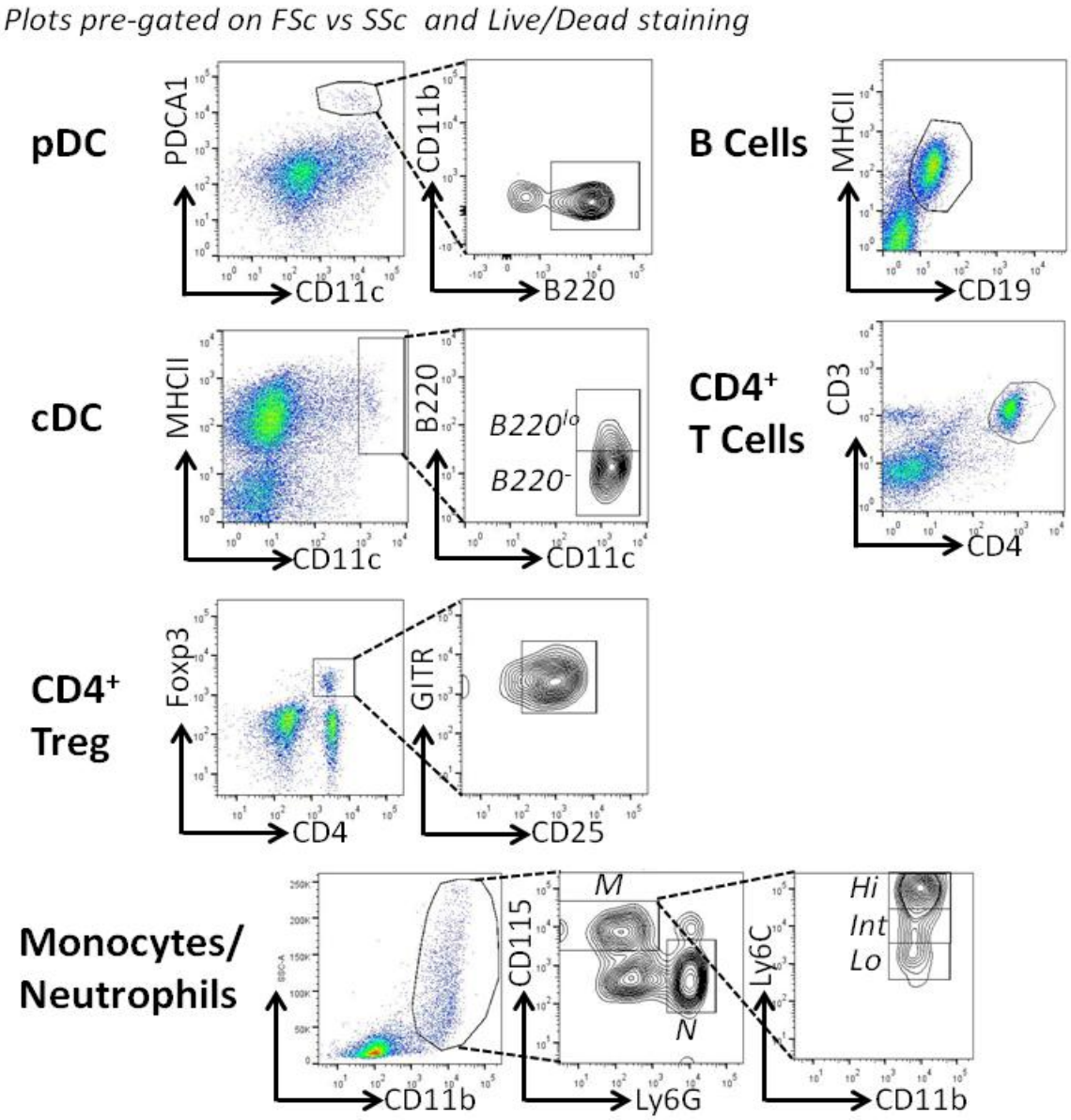
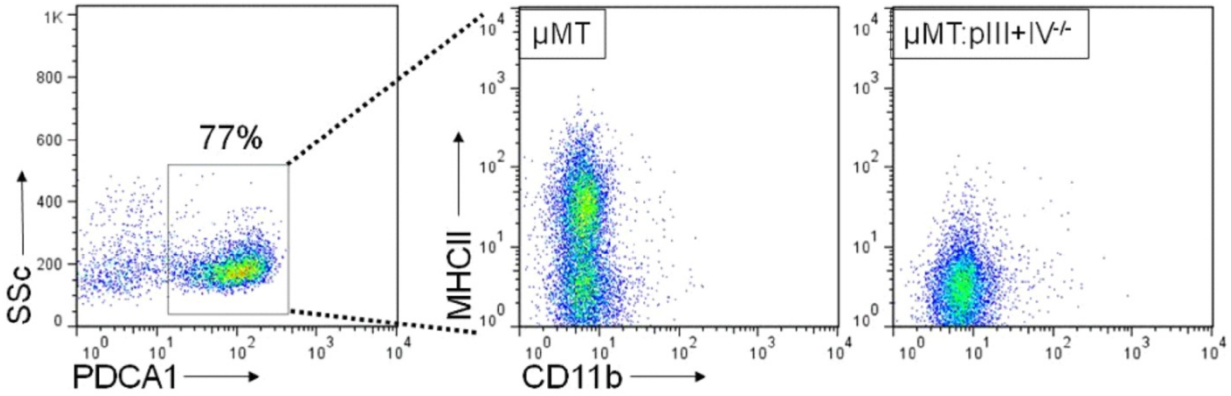
Figure S1



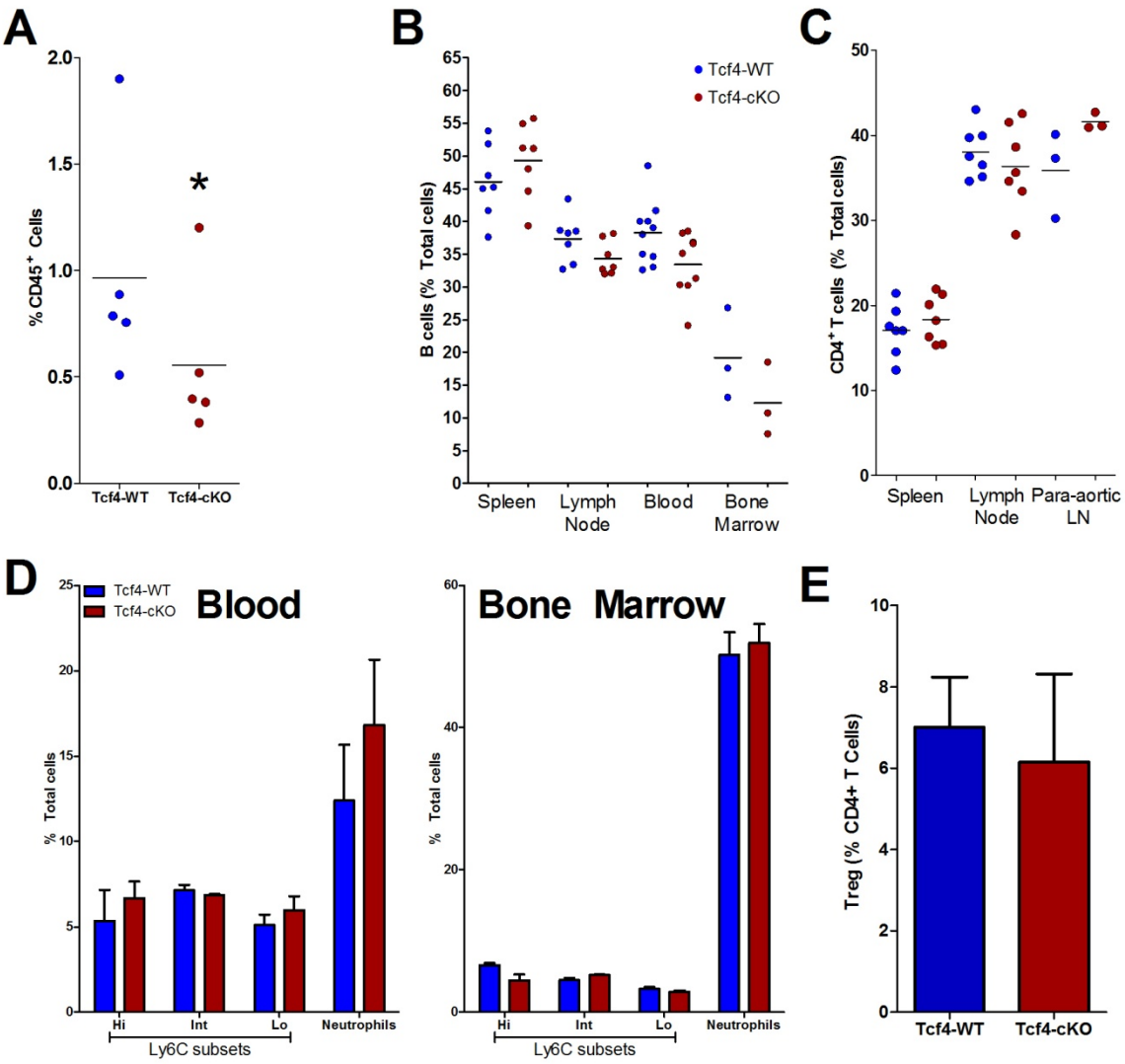
Supplemental Figure 1. Representative plots of gating strategies used. Plasmacytoid dendritic cells (pDCs): CD11c^{+} PDCA1^{+} CD11b^{-} B220^{+}. This population is also SiglecH^{+} (98.1±1.0 %) and CD8^{+} (88.7±1.7). Conventional dendritic cells (cDCs) were defined as CD11c^{hi} MHCII^{+} and either B220^{lo} or negative. B cells were defined as CD19^{+} MHCII^{+}, CD4^{+} T cells as CD3^{+} CD4^{+} and regulatory T cells as CD4^{+} Foxp3^{+} CD25^{+} GITR^{+}. Monocytes were defined as CD11b^{+} CD115^{+} and Ly6C high, intermediate or low. Neutrophils were CD11b^{+} Ly6G^{+} Ly6C^{+}.

Figure S2



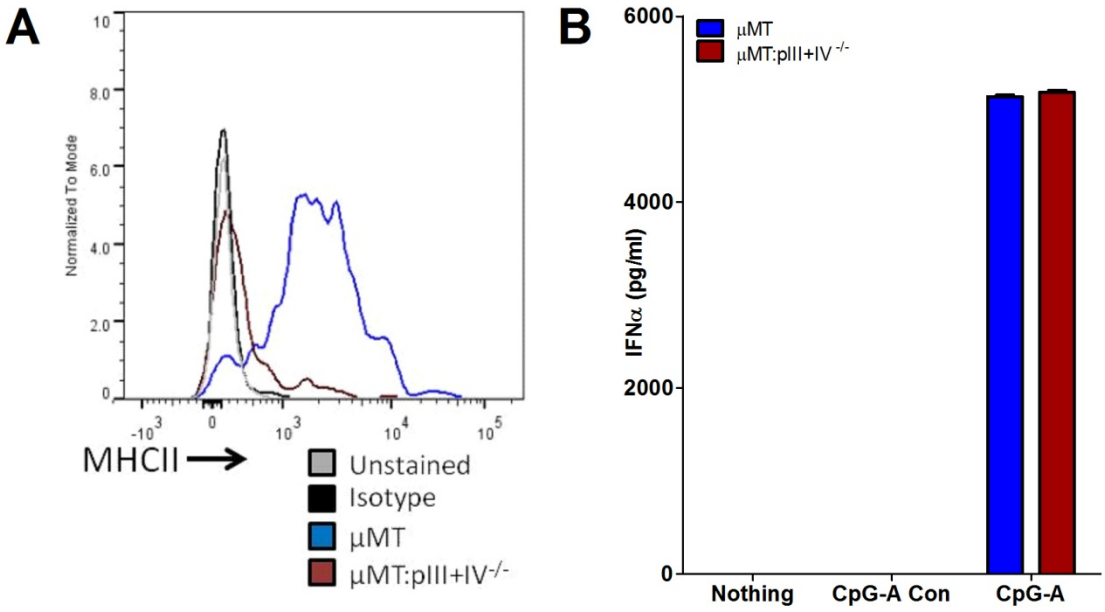
Supplemental Figure 2: Purity of bone marrow pDCs isolated by negative magnetic selection and expression of MHCII in µMT or µMT:pIII+IV$^{-/-}$ pDCs.

Figure S3



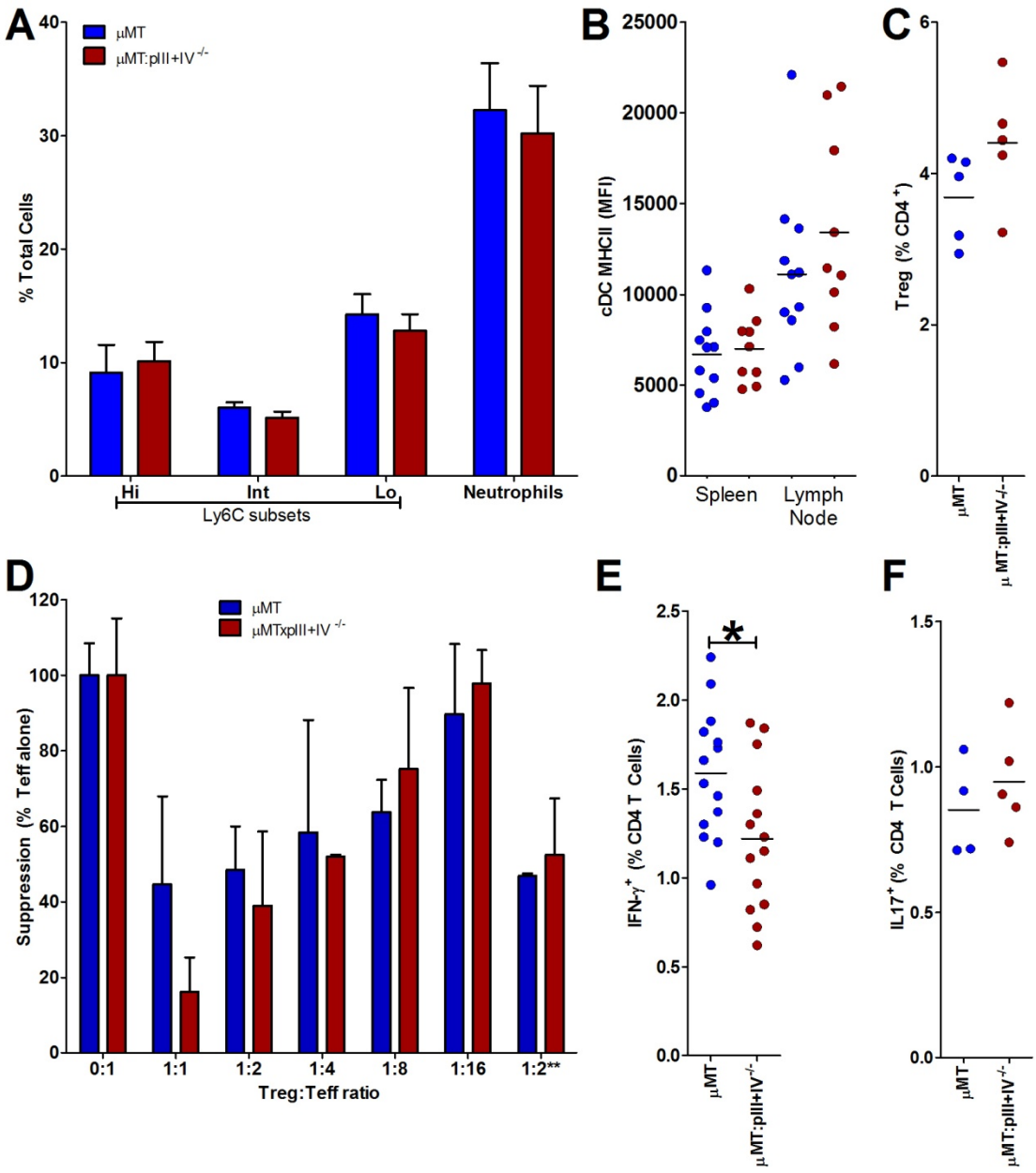
Supplemental Figure 3. Plasmacytoid dendritic cell, lymphocyte and monocyte levels in *Ldlr–/–* mice transplanted with *Tcf4-WT or Tcf4-cKO* mice. *p<0.05 vs WT. Data representative of 2 separate experiments. **A**. Aortic pDCs. **B**. B cells. **C**. CD4+ T cells. **D**. Ly6C monocyte subsets and neutrophils in blood and bone marrow (BM). **E**. Spleen regulatory T cells.

Figure S4



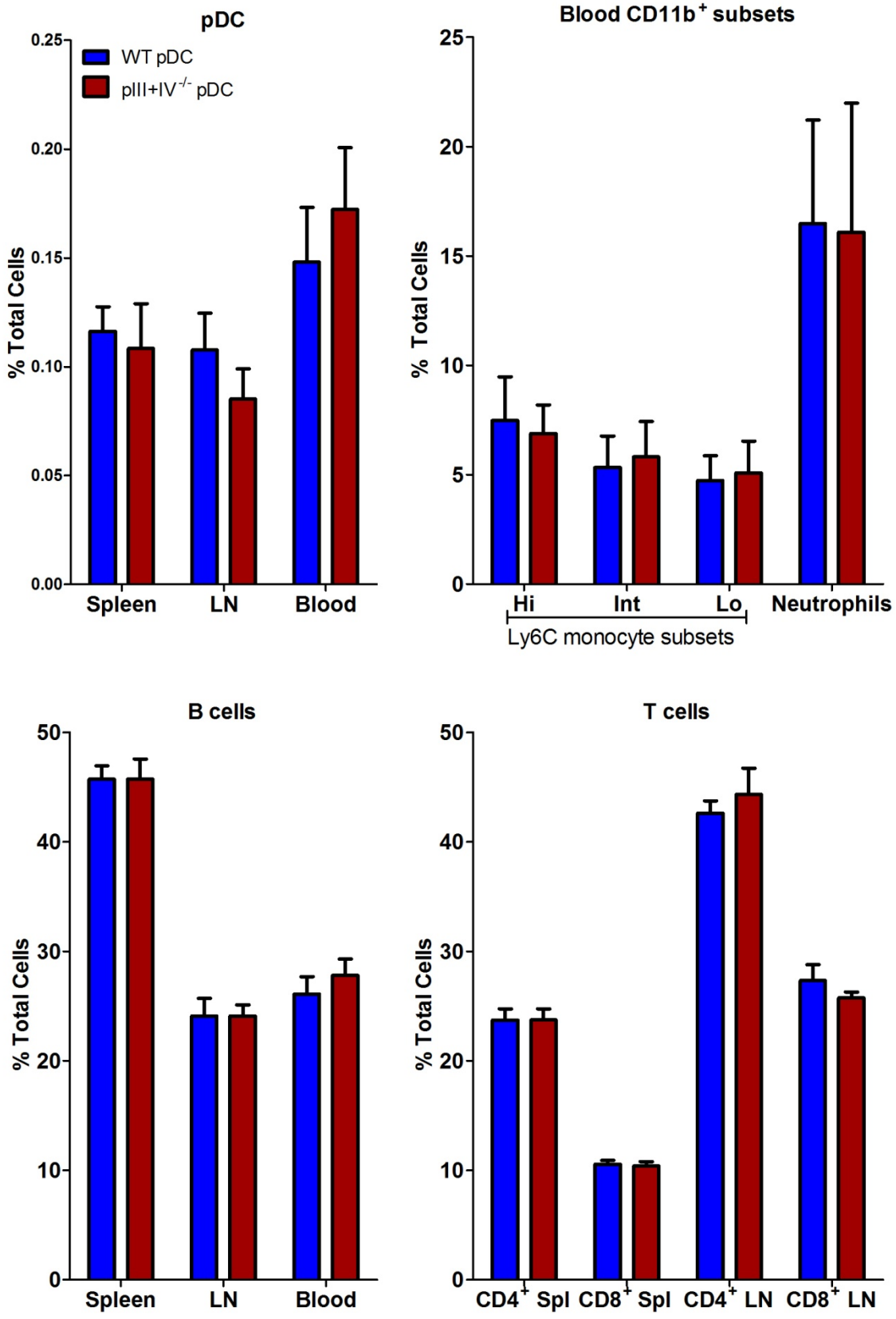
Supplemental Figure 4. **A**. Anti-MHCII staining on pDCs from µMT or µMT:pIII+IV $^{-/-}$ mice compared to isotype control staining. **B**. IFNα levels in conditioned medium from bone marrow pDCs from µMT or µMT: *pIII+IV*$^{-/-}$ mice untreated or treated with CpG control (GpC) or CpG -A (10µg/ml) for 24h. Data representative of 2 separate experiments performed in triplicates.

Figure S5



Supplemental Figure 5 . Immune cell levels and functions in Ldlr$^{-/-}$ mice reconstituted with μMT or μMT:pIII+IV$^{-/-}$ bone marrow. **A**. Ly6C monocyte subsets and neutrophils in blood and bone marrow (BM) . Data representative of 3 separate experiments **B**. MFI for MHCII in cDCs. Pooled data from 2 experiments. **C**. Spleen regulatory T cells. Representative of 3 separate experiments. **D**. Suppressive capacity of spleen CD4^{+} CD25+ regulatory T cells on CD4^{+} CD25^{-} effector T cell proliferation to anti -CD3 in the presence of WT spleen CD11c^{+} cells expressed as % of effector T cell proliferation alone. Representative of one experiment performed in triplicates with cells pooled from 5 animals/group. **E**. IFN-γ^{+} CD4^{+} T cells. Pooled data from 3 experiments. **F**. IL-17^{+} CD4 T cells. Representative of 3 experiments.

Figure S6



Supplemental Figure 6. Lymphoid tissue and blood levels of pDCs (A), monocytes and neutrophils (B), B cells (C) and T cells (D) in *Ldlr–/–* mice reconstituted with µMT (80%)/WT (20%) or µMT: *pIII+IV*$^{-/-}$ (80%)/ WT (20%) bone marrow. See methods for gating strategy. Data pooled from 2 experiments with at least 5 animals per experiment and per group.

Table S1. Flow Cytometry antibodies

Target	Clone	Company
Ly6C	7/4	AbD Serotec
CD45	30-F11	BD
Ly-6G	1A8	BD
CD11b	M1/70	Biolegend
CD25	PC61	Biolegend
PDCA1	927	Biolegend
CD44	1M7	Biolegend
CD62L	MEL-14	Biolegend
CD69	H1.2F3	Biolegend
CD8a	53-6.7	Biolegend
GITR	DTA-1	Biolegend
IFN-γ	XMG1.2	Biolegend
IL-10	JES5-16E3	Biolegend
IL-17A	TC11-18H10.1	Biolegend
MHC II	M5/114.15.2	Biolegend
CD3	145-2C11	Biolegend
B220	RA3-6B2	eBioscience
CD11c	N418	eBioscience
CD4	RM4-5	eBioscience
CD40	1C10	eBioscience
Foxp3	FJK-16a	eBioscience
IgM	II/41	eBioscience
Y-Ae	eBioY-Ae	eBioscience
CD86	PO3.3	Miltenyi
SiglecH	551.3D3	Miltenyi