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Materials and Methods

T cell proliferation assay

$CD4^+$ *naïve* T cells from OVA-TCR transgenic DO11.10/$RAG2^{-/-}$ mice were labeled with 2 μM Carboxyflourescein diacetate succinimidyl ester (CFSE-Molecular Probes) in PBS for 15 min. sDCs (2 x 10^4) stimulated or not with PAM (100 ng/ml) were cultured with labeled $CD4^+$ T cells (1 x 10^5) in the presence of 0.01, 1.0 or 10 μM OVA in a total volume of 200 μl in 96-well plates for 4 days.

Blocking experiments

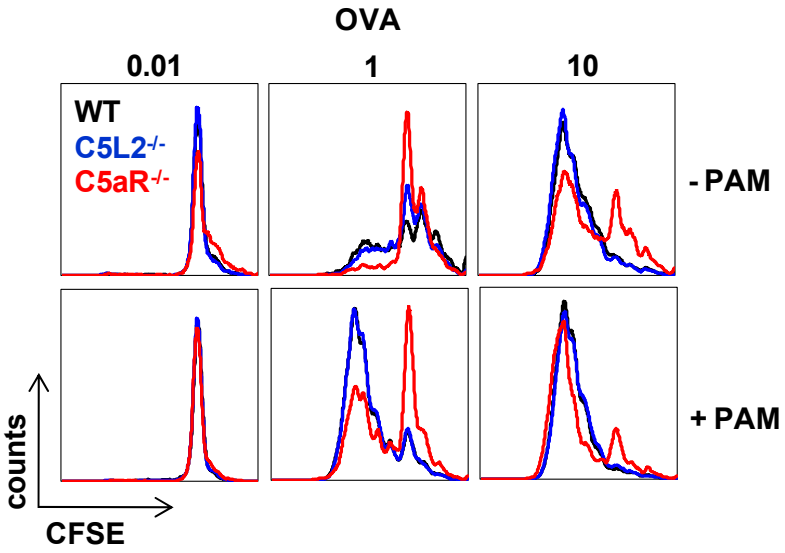
DCs (2 x 10^4) incubated with and without PAM_3CSK_4 were cultured for 4 days with $CD4^+$ T cells (1 x 10^5) in a total volume of 200 μl in 96-well plates. Cultures were performed in the presence of 1 μM OVA (Sigma) as indicated. Where indicated anti-IL-6 (MPS-20F3, eBiosciences), anti-TGF-β (1835, R&D Systems), anti-IL-21 (AF594, R&D Systems), anti-IL-23 (G23-8, EBiosciences) or the appropriate isotypes were used at 10 μg/ml.

Cell extracts and immunoblot assay

sDCs (2 x 10^6) were treated with C5a (10 nM) for different time periods. C5aRA (10 μM) was included where indicated. The reaction was stopped by the addition of cold PBS/orthovanadate (2 mM) at each time point. Cells were incubated for 5 min on ice in lysis buffer (1% Triton X-100, 5 mM EDTA, 50 mM NaF, 10 mM Tris/Cl [pH7.6], 0.1% BSA, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were cleared by centrifugation, applied to 12% SDS PAGE and analyzed by Western blotting using anti-phosphorylated ERK (BD Biosciences) and anti-rabbit IgG HRP (Chemicon International). Stripping of the blots (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM tris-HCl pH 6.7 for 30 min at 50^{o}C) was performed in order to quantify total ERK (anti-ERK- BD Biosciences). Signals were visualized by ECL detection system (GE Healthcare).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was harvested using Trizol® Reagent (Invitrogen) according to manufacturer's instructions. Residual genomic DNA was removed by incubation with RNase-free DNase (Sigma). RNA (1 μg) was converted to cDNA using the SuperScript™ II Reverse Transcriptase (Invitrogen). cDNAs were amplified by PCR (40 cycles: 94°C/1min, 58°C/1min, 72°C/1min) in the presence of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 0.4 mM dNTPs and 0.8 pmol of each specific primer. Amplification of 18s rRNA was used as a control. The primer sequences are as follows. C5aR: sense 5'-GGTCTCTCCCCAGCATCATA-3'; antisense 5'-GGCAACGTAGCCAAGAAAAA-3', C5L2: sense 5'-CTGGGCCTCTTGCTGACTGTGC3'; antisense 5'- GCCCCAGGAAGCCAAAGAGGA-3' and 18s rRNA: from SuperArray Bioscience Corporation-reference n° K01364. Amplification products were analyzed in a 3.0% agarose gel followed by ethidium bromide staining.



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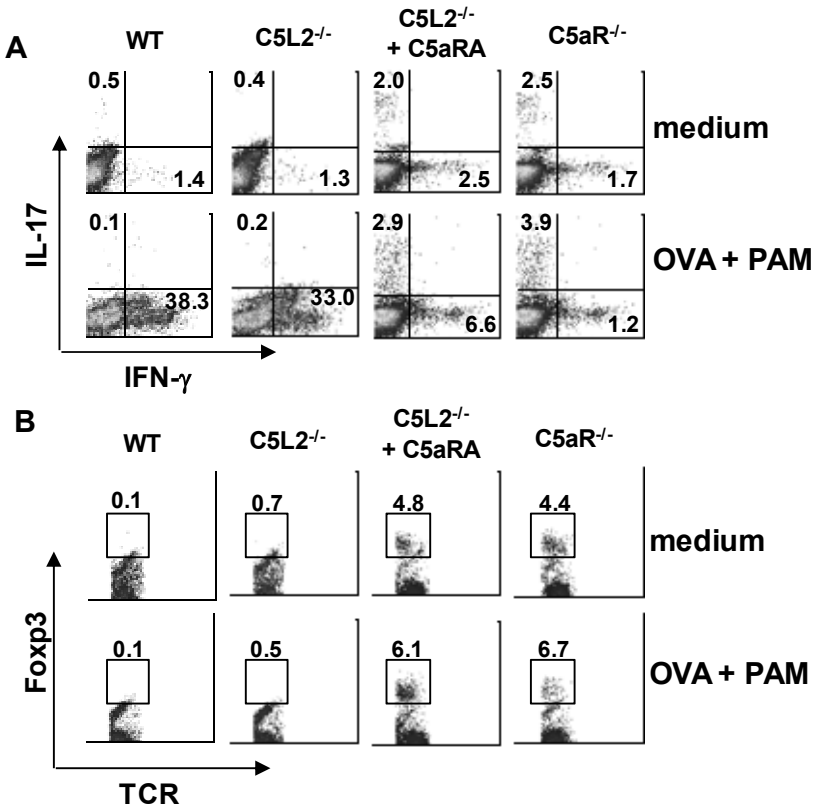
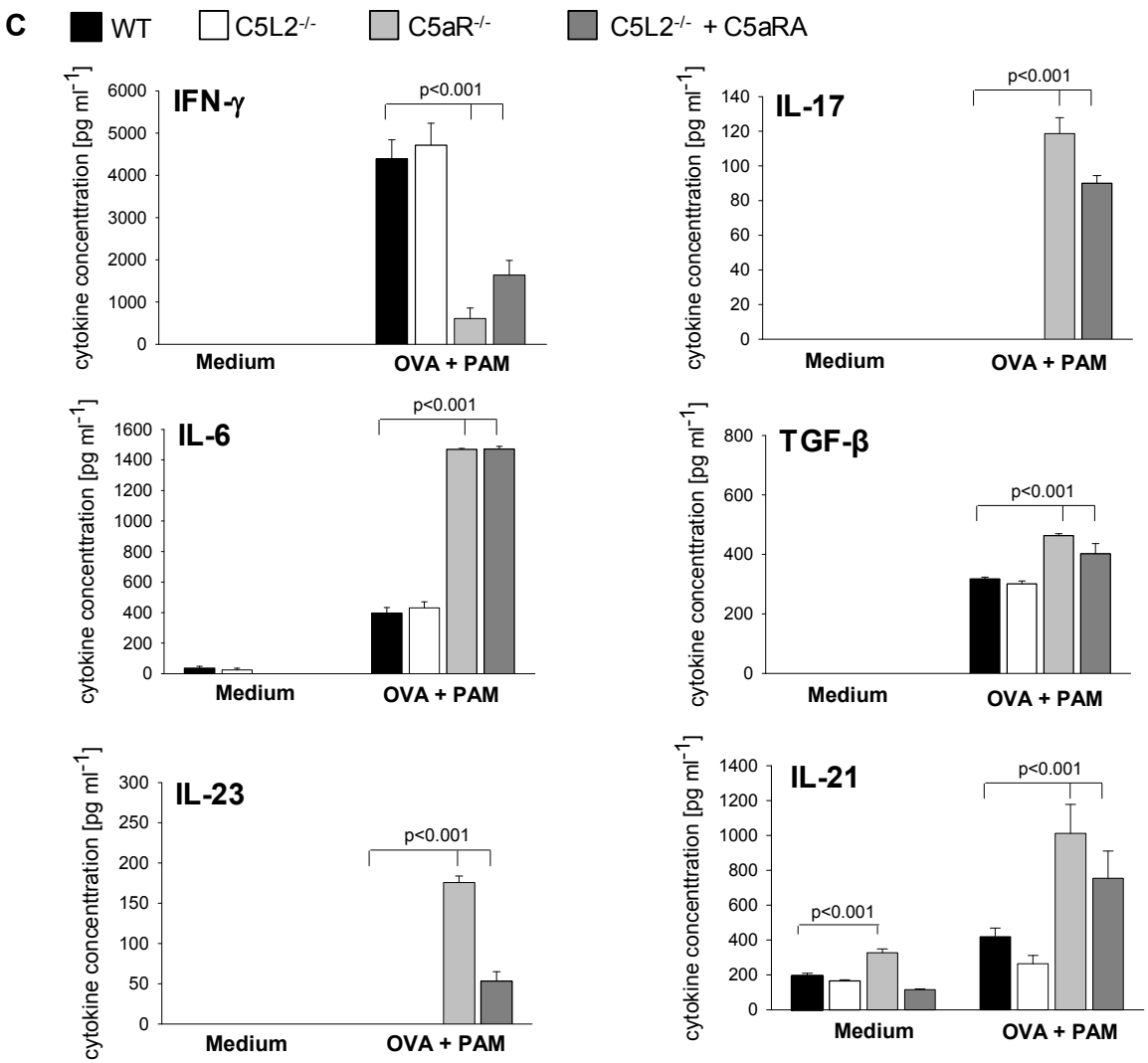
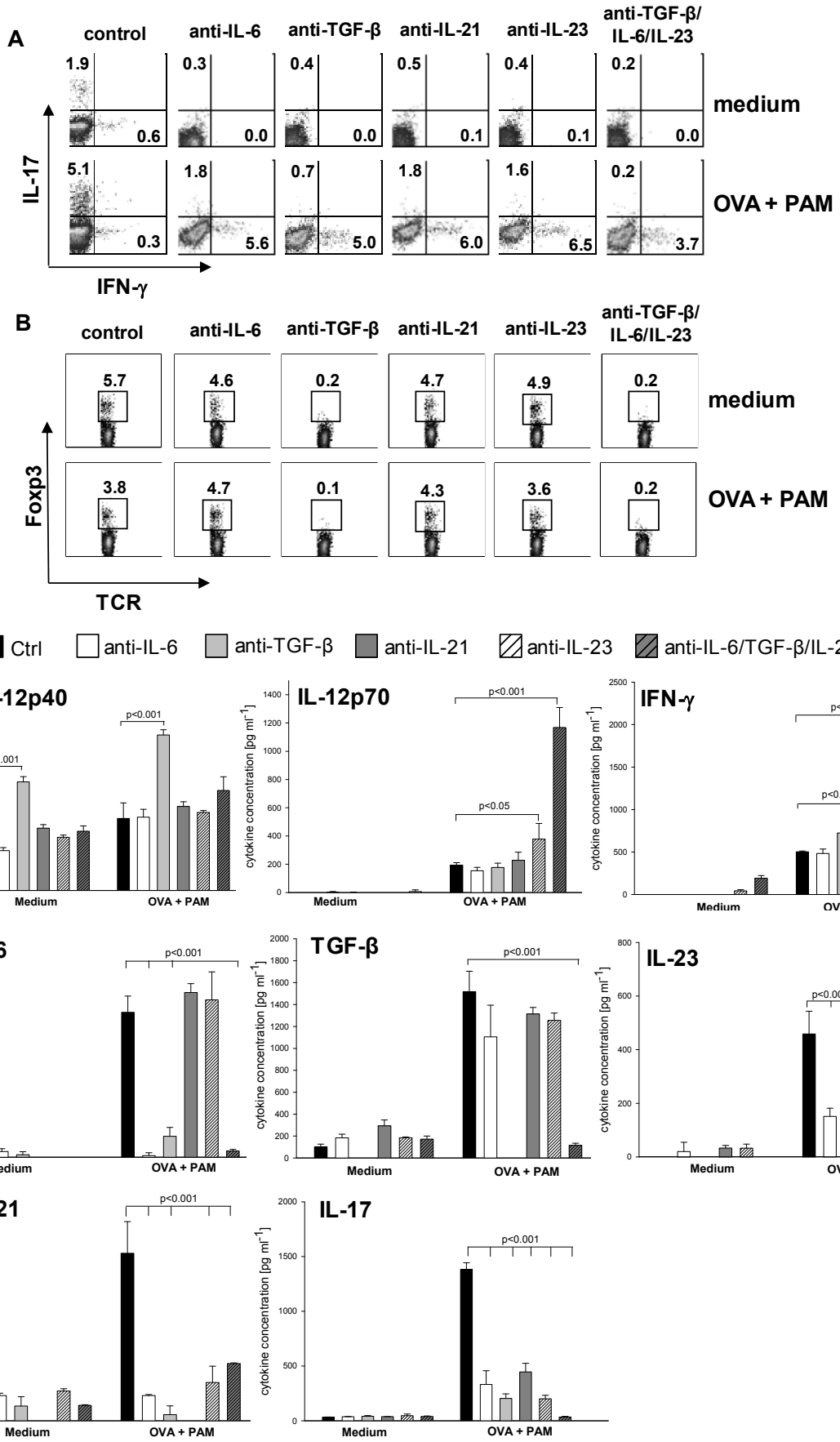
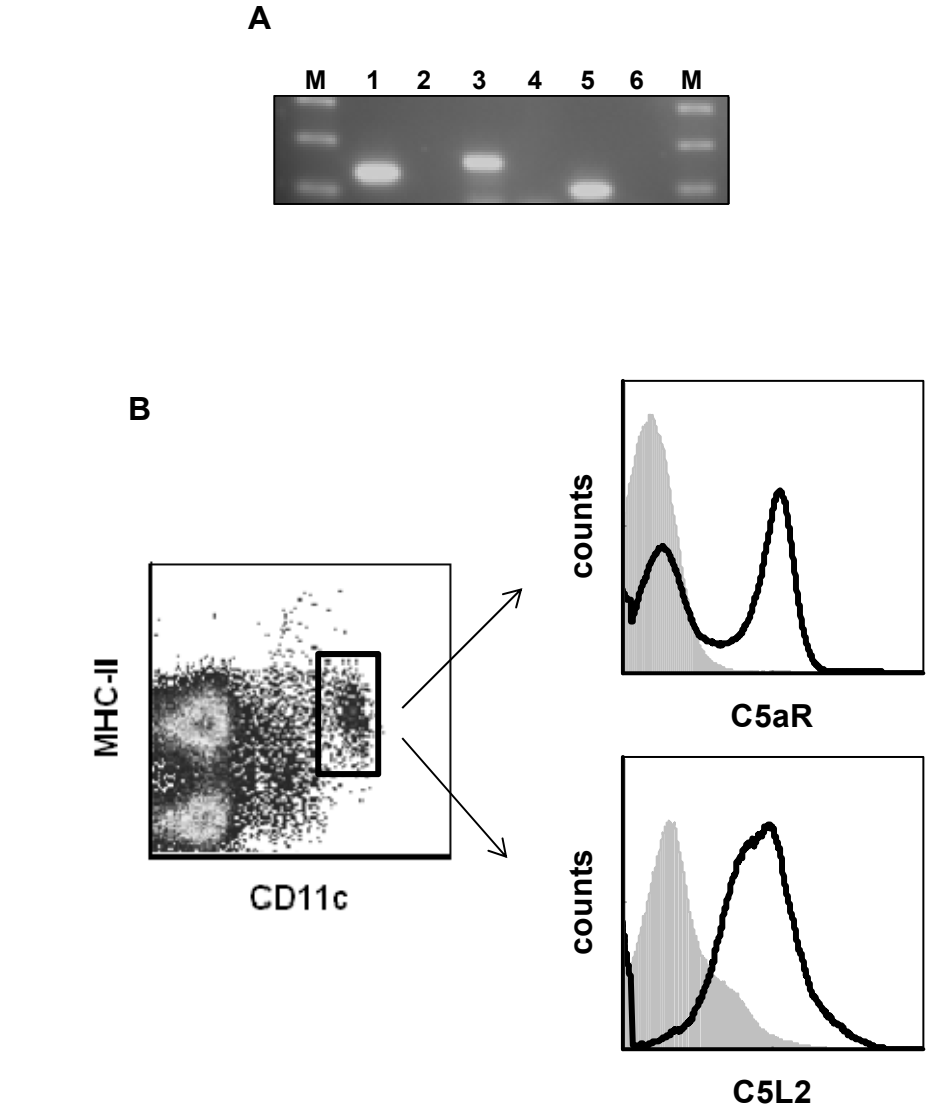




Supporting Information Fig.2 Weaver et al.

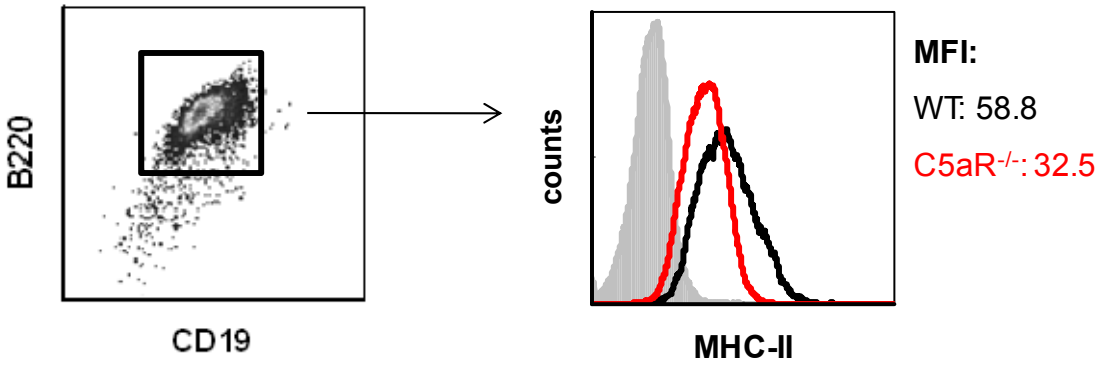


Supporting Information Fig.3 Weaver et al.



Supporting Information Fig.4. Weaver et al.

A



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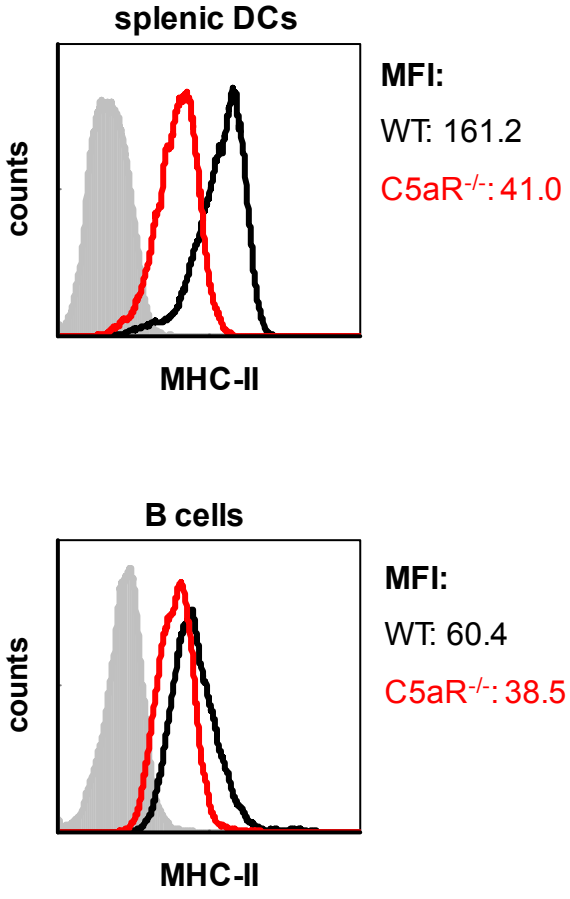

Supporting Information Fig. 5 Weaver et al.

Supporting Information- Figure Legends

Figure 1. C5aR$^{-/-}$ sDCs have an impaired ability to promote CD4^{+} T cell proliferation. sDCs from WT, C5aR$^{-/-}$, and C5L2$^{-/-}$ mice were co-cultured with naïve CD4^{+} T cells from OVA-TCR transgenic DO11.10/RAG2$^{-/-}$ mice for 4 days in the absence or the presence of PAM. OVA was added to the cell cultures at the indicated concentrations. CD4^{+} T cells were labeled with 2 μM carboxyflourescein diacetate succinimidyl ester (CFSE) and the number of CFSE^{+} CD4^{+} T cells was analyzed by flow cytometry.

Figure 2. C5aR signaling in sDCs promotes Th17 differentiation even under Th1 conditions. (A) sDCs from WT, C5aR$^{-/-}$, and C5L2$^{-/-}$ mice were co-cultured with naïve CD4+ T cells from OVA-TCR transgenic DO11.10/RAG2$^{-/-}$ mice for 4 days and challenged with OVA (1 μM) in the absence or presence of PAM. rIL-12 (10 ng) and anti-IL-4 (10 μg/ml) were added to induce Th1 differentiation. The frequencies of IL-17A^{+} and IFN-γ^{+} CD4^{+} or (B) Foxp3^{+} CD4^{+} T cells were determined by intracellular staining using flow cytometry. C5aR antagonist was added to some cultures as indicated. Data are representative of 3 independent experiments. (C) Concentrations of IFN-γ, IL-17A, IL-6, TGF-β, IL-23 and IL-21 in culture supernatants from (A) as determined by ELISA. Data are mean ± s.d. (n=3).

Figure 3. TGF-ß is critical for C5aR-dependent Th17 differentiation sDCs from C5aR$^{-/-}$ mice were co-cultured with naïve CD4^{+} T cells from OVA-TCR transgenic DO11.10/RAG2$^{-/-}$ mice for 4 days in the absence or presence of PAM. Neutralizing antibodies to IL-6, TGF-β, IL-21, IL-23, or the combination of anti-IL-6, anti-TGF-β and anti-IL-23 (all at 10μg/ml) were added to the co-cultures. The frequencies of (A) IL-17A^{+} and IFN-γ^{+} CD4^{+} T cells were determined by intracellular staining using flow cytometry. The control is representative of the isotype controls used for each experiment. (B) As in (A), except that Foxp3^{+} CD4^{+} T cells were determined by intracellular staining using flow cytometry. (C) Concentrations of IL-12, IFN-γ, IL-6, TGF-β, IL-23, IL-21 and IL-17A in culture supernatants from (A) were determined by ELISA. Data are mean ± s.d. (n=3).

Figure 4. Cellular expression and functional properties of C5aR and C5L2 on sDCs. (A) Total RNA from $CD11c^+$ sorted sDCs was extracted, and mRNA levels of C5aR (lane 1), C5L2 (lane 3), and 18s rRNA (lane 5) were determined by RT-PCR. Lanes 2, 4 and 6 depict control samples without cDNA. M- corresponds to the molecular weight marker. (B) Surface expression of C5aR and C5L2 on MHC-$II^+/CD11c^{high}$ sDCs as determined by flow cytometry. Gray filled histograms depict isotype control. (C) sDCs from WT, $C5aR^{-/-}$, and $C5L2^{-/-}$ mice were incubated for the indicated times with C5a. Cell lysates were run on SDS-PAGE, and phosphorylated ERK1/2 was detected by Western blotting. Loading controls were assessed with antibodies to ERK1/2.

Figure 5. Low MHC-II expression levels in C5aR-deficient antigen presenting cells. (A) Surface expression of MHC-II on $B220^+/CD19^+$ splenic B cells from WT and $C5aR^{-/-}$ mice on the BALB/c background as determined by flow cytometry. (B) Surface expression of MHC-II on $CD11c^{high}$ sDCs and $B220^+/CD19^+$ splenic B cells from WT and $C5aR^{-/-}$ mice on the C57BL/6 background as determined by flow cytometry. Gray filled histograms depict isotype controls. MFI: median fluorescence intensity.

Supporting Information Table 1. $C5aR^{-/-}$ sDCs have an impaired ability to promote $CD4^+$ T cell proliferation.

	Proliferating DO11.10 $CD4^+$ T Cells (%)		
OVA (μM)	**0.01**	**1.0**	**10**
WT	4	32	85
$C5aR^{-/-}$	4	10	59
$C5L2^{-/-}$	4	27	87
OVA (μM) + PAM_3CSK_4	**0.01**	**1.0**	**10**
WT	4	81	92
$C5aR^{-/-}$	3	50	79
$C5L2^{-/-}$	4	81	90

Spleen derived $CD11c^+$ sorted sDCs from WT, $C5aR^{-/-}$, and $C5L2^{-/-}$ mice were co-cultured with naïve $CD4^+$ T cells from OVA-TCR transgenic DO11.10/$RAG2^{-/-}$ mice in the absence or presence of PAM_3CSK_4 (100ng/ml). Ovalbumin was added to the cell cultures at increasing concentrations. $CD4^+$ T cells were labeled with 2 μM carboxyflourescein diacetate succinimidyl ester (CFSE) and the numbers of $CFSE^+$ $CD4^+$ T cells were analyzed by flow cytometry after 4 days of co-culture.