Supporting Information

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SI Materials and Methods

Cells. pDCs were purified by BDCA-4 magnetic bead separation (Miltenyi Biotec) after density centrifugation on Ficoll. The pDC line GEN (1, 2) was cultured with the MS-5 feeder cell line in RPMI 1640 (GlutaMAX; Invitrogen) supplemented with sodium pyruvate, gentamicin, nonessential amino acids, and 10% FCS (vol/vol) (Invitrogen). Naive $CD4^+$ T cells were purified in two steps from peripheral blood mononuclear cells. Naive $CD4^+$ T cells were enriched using the Naive CD4 Isolation Kit (Miltenyi Biotec). After enrichment, cells were stained, and naive cells were sorted with a FACSAria cell sorter (BD Biosciences) as cells negative for CD8, CD56, CD11c, CD14, CD25, CD45RO, CD123, CD19, and HLA-DR and positive for CD45RA. The sorted cells were more than 98% $CD4^+CD45RA^+$. Monocyte-derived dendritic cells (moDCs) were differentiated as described previously (2). Monocytes were purified from peripheral blood mononuclear cells by 2 h of adherence and cultured with 300 U of IL-4/mL (Immunex) and 100 IU of GM-CSF/mL (Schering-Plough) for 5 d to obtain immature moDCs.

HIV and TLR Agonist Stimulation. Primary pDCs were stimulated for 24 h by 30 ng/mL p24 equivalent of HIV laboratory strains (SF162) or infectious molecular clones (JR-CSF, CH058, CH077, or CH106), obtained through the Center for HIV-AIDS Immunology. pDCs or the GEN line were stimulated by 2 μg/mL of CpG oligonucleotide (GGGGACGACGTCGTGGGGGGG) (Integrated DNA Technologies) or 0.5–1 μM resiquimod (R848; 3M). The GEN line was rested away from MS-5 cells for 2–3 d before stimulation, which was found to enhance its stimulation by TLR.

IDO Detection. RNA of cells was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The following primers were used for RT-PCR: for IDO, 5′-GCGCTGTTGGAAATAGCTTC-3′ (forward), 5′-CAGGACGT- CAAAGCACTGAA-3′ (reverse); for β-actin, 5′-TGACCCAGATCAT- GTTTGAGA-3′ (forward), ACTCCATGCCCAGGAAGGA-3′ (reverse). IDO protein was detected by Western blot analysis using rabbit polyclonal IDO antibody (Axxora). Band intensity was quantified using ImageQuant TL software (Amersham Biosciences).

p50 and p52 Nuclear Translocation. GEN cells were stimulated by R848 for 1–6 h, with or without a 1-h preincubation with 50 μM NEMO-binding peptide (NBD), either WT (drqikiwfqnrrmkwkk-TALDWSWLQTE) or mutant (drqikiwfqnrrmkwkkTALDASALQTE) (3). Cytosolic and nuclear fractions were extracted using the Active Motif Nuclear Extract Kit. p50 and p52 nuclear translocation was measured using Active Motif TransAM p50 and p52 Kits.

siRNA Knockdown. GEN cells were rested without feeder cells for 1 or 2 d, before transfection by ON-TARGETplus SMARTpool siRNA (Dharmacon) for NIK (MAP3K14), IKK-α (CHU.K.), TLR-7, TLR-9, or control siRNA (ON-TARGET plus GAPDH control), using an Amaxxa nucleofector. After 12–24h, cells were stimulated by R848 (1 μM).

Activation of the Noncanonical NF-κB Pathway. Rabbit antibodies directed against IKK-α, p100, phospho-p100, phospho-IKK-α/β, NIK, and TRAF3 were purchased from Cell Signaling Technology.

Immunoprecipitation. Cells were stimulated for various time points and lysed in M-Per Mammaian Protein Extraction Reagent (Thermo Fisher Scientific). Proteins were immunoprecipitated using anti-MyD88 (Santa Cruz Biotechnology), anti-NIK (Cell Signaling Technology), and anti–IKK-α (Cell Signaling Technology) and Pierce Protein G Agarose. Western blot analyses were performed using rabbit anti-MyD88 (Cell Signaling Technology), rabbit anti-TRAF3 (Cell Signaling Technology), or mouse anti-TRAF3 (Santa Cruz Biotechnology).

Luciferase Reporter Assay. The pNiFty-Luc plasmid from Invivogen encodes for the firefly luciferase downstream of the proximal ELAM promoter combined with five NF-κB sites (GGGGACTTTCC). The region encoding the five NF-κB sites was exchanged for the five copies of the full noncanonical binding sites (GGGAGATTTG) (4), or for five copies of the partial noncanonical binding sites in the IDO upstream region (GGGAGACAGA), between the EcoRI and PstI restriction sites. Cells were electroporated using an Amaxxa nucleofector, and then stimulated by R848 or CpG. Luciferase activity was read after 4 h and 18 h.

ChIP. GEN cells were left unstimulated or were stimulated with R848 for 6 h, after which they were fixed in formaldehyde. Chromatin was sonicated down to 200- to 500-bp fragments for immunoprecipitation of RelB (Active Motif) using the MAGnifyChromatin Immunoprecipitation System (Invitrogen).

Treg Induction and T-Cell Suppression Assay. Naive $CD4^+$ T cells were enriched using the Naive CD4 Isolation Kit (Miltenyi Biotec). After enrichment, cells were stained and naive cells were sorted on a FACSAria (BD) as cells negative for CD8, CD56, CD11c, CD14, CD25, CD45RO, CD123, CD19, and HLA-DR and positive for CD45RA. Sorted cells were more than 98% $CD4^+CD45RA^+$.

Treg induction was performed as described previously (1). pDCs were stimulated by R848 (0.1 μM or 1 μM) or CpG oligonucleotide (2 μg/mL), washed, and added to naïve $CD4^+$ T cells (1:10 pDC: T-cell ratio). T cells were collected at day 7 and bead-purified using CD25 beads from Miltenyi Biotec.

For inhibition of moDC maturation, $CD25^+$ cells were added to pDC-autologous moDCs (1:1 ratio) in the presence of 1 μg/mL anti-CD3 (NA/LE; BD Biosciences) for 1 d, followed by stimulation of moDCs with LPS (100 ng/mL). Supernatant was collected after overnight stimulation, and TNF-α levels were measured by cytokine bead array (BD Biosciences). Blocking anti–CTLA-4 (ANC152.2; Ancell) or IgG1 isotype (BD Biosciences) was added at 5 μg/mL before addition of the T cells.

For IDO expression, $CD25^-$ or $CD25^+$ cells were added to moDCs for 2 d in the presence of 1 μg/mL of anti-CD3. Cells were washed extensively, and RNA was extracted on adherent cells for measurement of IDO expression by RT-PCR.

CTLA-4–Induced IDO Expression and Treg Generation. moDCs were incubated for 3 d with CTLA-4-Ig (40 μg/mL; Chimerigen) or pooled human IgG (ChromPure Human IgG, whole molecule; Jackson ImmunoResearch), after which RNA was extracted for IDO measurement by RT-PCR. For generation of Tregs, CTLA-4-Ig was added to moDCs for 1 d. A mixture of 5 ng/mL of TNF-α (R&D Systems), 5 ng/mL of IL-1β (R&D Systems), and 1 μg/mL of prostaglandin E2 (Sigma-Aldrich), or LPS (100 ng/mL), was added for an additional day, after which naïve $CD4^+$ T cells were added to the moDCs (1:10 ratio) for 6–7 d in the presence or absence of 200 μM 1-methyl tryptophan. $CD25^+$ cells were isolated using CD25 beads from Miltenyi Biotec. For suppression of naïve T-cell proliferation, $CD25^+$ T cells were added to autologous naïve $CD4^+$ T cells (1:1 ratio) labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE), with plate-bound anti-CD3 and anti

CD28 [10 μg/mL of anti-CD3 (NA/LE; BD Biosciences) and 20 μg/mL of anti-CD28 (BD Biosciences)] for 5–7 d. Proliferation of CFSE-labeled T cells ($CFSE^{high}$ cells) was measured on a FACS-Calibur machine (BD Biosciences).

1. Jacquelin B, et al. (2009) Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response. *J Clin Invest* 119:3544–3555.
2. Fonteneau JF, et al. (2004) Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *J Virol* 78(10):5223–5232.
3. Belladonna ML, et al. (2008) Cutting edge: Autocrine TGF-beta sustains default tolerogenesis by IDO-competent dendritic cells. *J Immunol* 181:5194–5198.
4. Bonizzi G, et al. (2004) Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *EMBO J* 23:4202–4210.

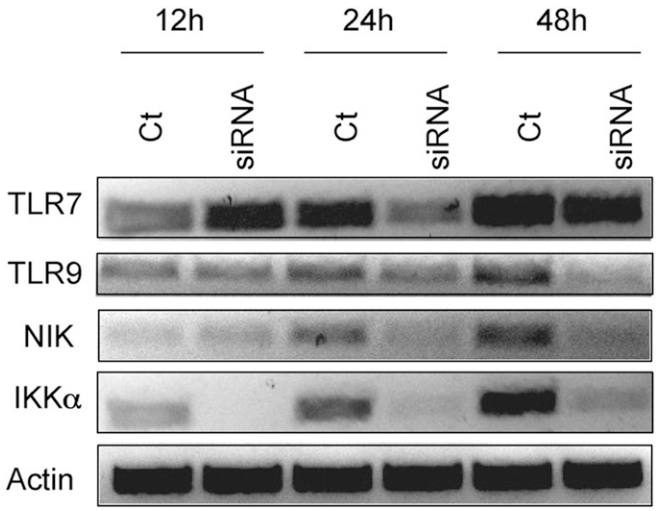

Fig. S1. Kinetics of siRNA knockdown of TLR-7, TLR-9, NIK, and IKK-α. GEN pDCs were rested for 2 d in medium alone, before siRNA nucleofection (Amaxxa) with 1 nmol siRNA specific for TLR-7, TLR-9, NIK, IKK-α, or control siRNA (all from Dharmacon). RNA was extracted at 12 h, 24 h, and 48 h, and expression of the target genes was measured by RT-PCR. One representative β-actin expression (from the TLR-7 knockdown time course) is displayed as a control. Viability of the cells after nucleofection varied from experiment to experiment, but was greatly reduced by 72 h (at least 50% cell death), as was responsiveness to TLR stimulation. Based on the knockdown kinetics, cells were stimulated at 24 h after siRNA nucleofection. Expression of TLR-7, TLR-9, NIK, and IKK-α was augmented over time on culture in medium alone (without feeder cells), and increased responsiveness to TLR-7 and TLR-9 stimulation was also seen (as measured by TNF-α secretion).

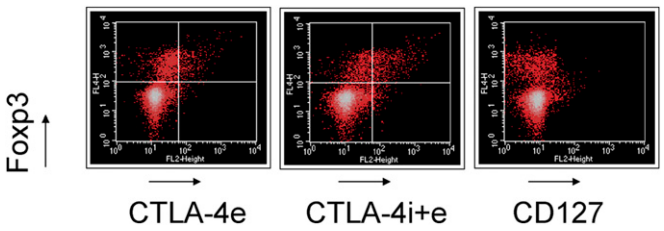

Fig. S2. CTLA-4 expression by $Foxp3^+$ cells. $CD4^+$ T cells stimulated for 7 d by R848-stimulated pDCs were analyzed by flow cytometry for expression of CTLA-4. Cells were stained for CTLA-4 without permeabilization (CTLA-4e) or with permeabilization (CTLA-4i+e), followed by staining for Foxp3. Quadrant bars indicate the position of cells on staining with isotype controls. CD127 staining was also performed in combination with Foxp3. The $CD127^{low}$ $Foxp3^+$ phenotype is consistent with a Treg phenotype.

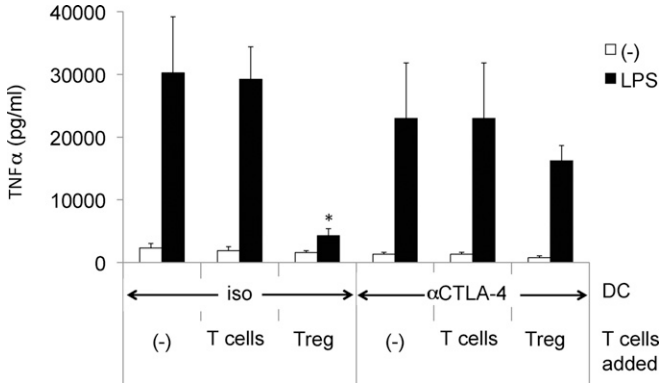

Fig. S3. CTLA-4 is partially responsible for inhibition of DC activation. $CD4^+$ T cells were stimulated for 7 d by CpG-stimulated (CpG) or unstimulated primary pDCs. $CD4^+CD25^+$ cells were then added ($CD25^+$) to pDC-autologous moDCs (1:1 ratio) for 1 d with CTLA-4 blocking antibody (CTLA-4) or isotype control (iso) at 5 μg/mL and anti-CD3 antibody at 1 μg/mL, after which cells were stimulated by 50 ng/mL of LPS. Secretion of TNF-α was measured after 18 h by cytokine bead array (*$P < 0.05$, Student *t* test).

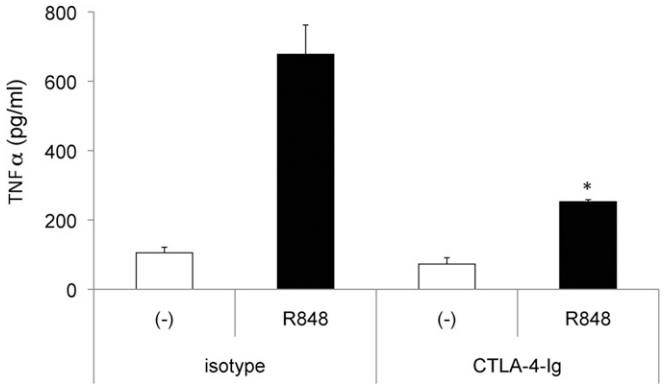

Fig. S4. CTLA-4-Ig inhibits cDC activation. moDCs were preincubated for 1 d with CTLA-4-Ig (40 μg/mL; Chimerigen) or pooled human IgG (ChromPure Human IgG, whole molecule, Jackson ImmunoResearch), and then stimulated by 0.5 μM R848. TNF-α was measured by cytokine bead array after overnight stimulation (*$P < 0.05$, Student *t* test).

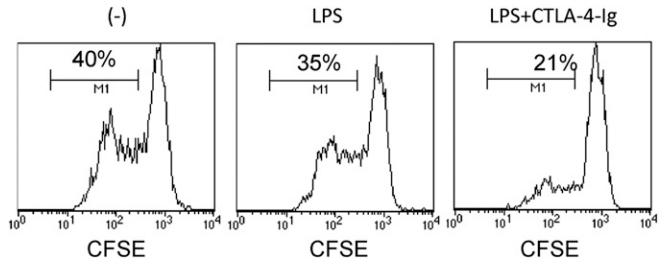

Fig. S5. T-cell proliferation is inhibited by CTLA-Ig–treated DC-induced Tregs. $CD4^+$ T cells were incubated with CTLA-4-Ig–treated moDCs for 7 d. Differentiated $CD4^+CD25^+$ cells from the cocultures were then added to CFSE-labeled naïve $CD4^+$ T cells (autologous to $CD4^+$ T cells from the DC T-cell culture; 1:1 ratio) in the presence of plate-coated CD3/CD28 antibodies. Proliferation was measured on $CFSE^+$ cells, and expressed as the percentage of $CFSE^{low}$ cells compared with undivided cells. An example of proliferation of naïve $CD4^+$ T cells alone (*Left*) is compared with naive $CD4^+$ T cells incubated with autologous $CD4^+$ T cells activated by LPS-activated DCs (*Middle*) and LPS-activated CTLA-Ig treated DCs (*Right*). Approximately 50% fewer naïve $CD4^+$ cells proliferate in the latter condition compared with the control condition.

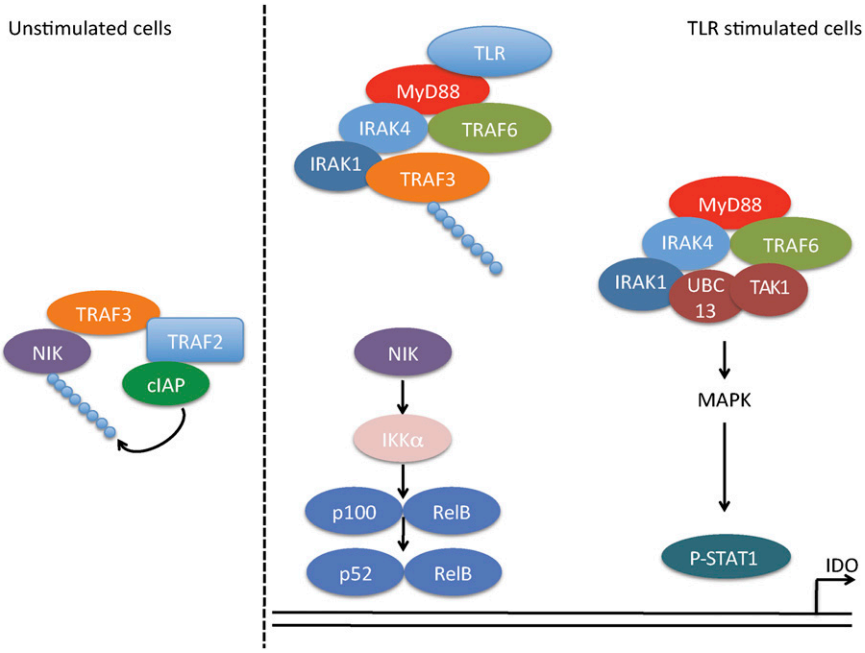

Fig. S6. Model for TLR-induced IDO induction. (*Left*) In unstimulated pDCs, NIK is constitutively ubiquitylated by the TRAF3/TRAF2/cIAP complex. (*Right*) On TLR stimulation, TRAF3 is recruited to the TLR/MyD88 complex, where it undergoes ubiquitylation and releases NIK. NIK can then activate IKK-α for activation of the noncanonical pathway. TRAF3 K48 ubiquitylation allows the release of MEKK1/TAK1-associated complex for MAPK activation, which may participate in STAT1 phosphorylation. Efficient IDO transcription is dependent on both p52/RelB and STAT1 binding to the *INDO* gene promoter.