Supporting Information

Onishi *et al*. 10.1073/pnas.0711106105

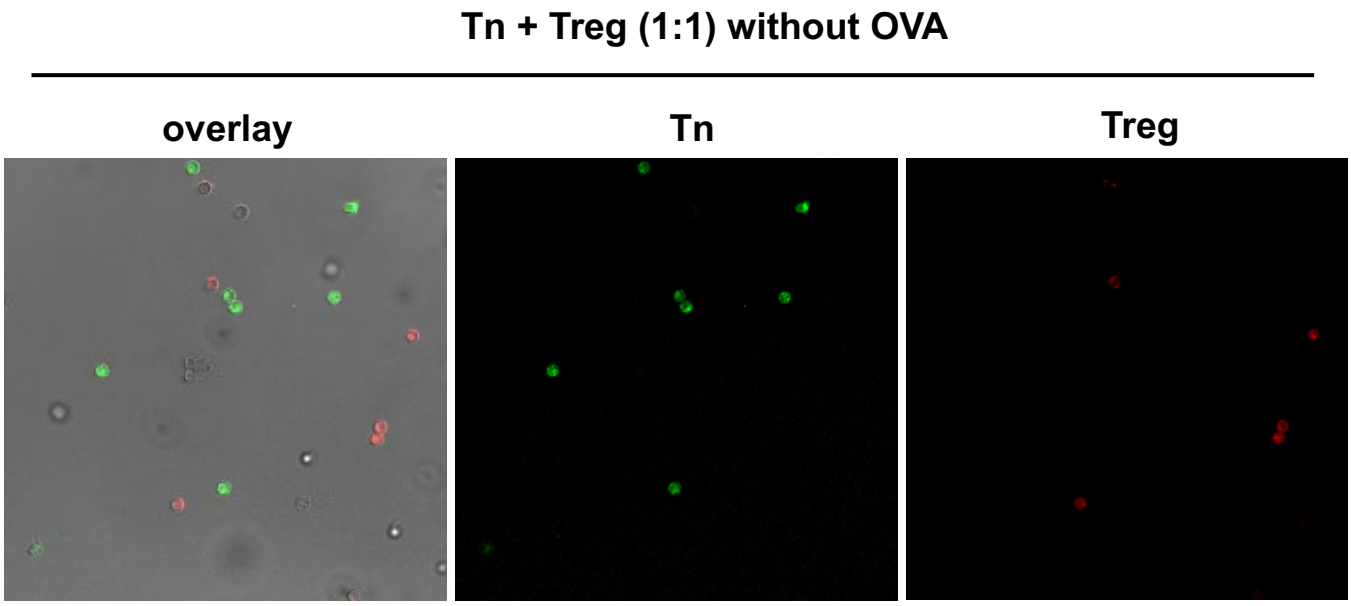

Fig. S1. Requirement of antigen for Treg aggregation. No aggregates were formed after the coculture of green-dye labeled naïve T cells, red-dye labeled regulatory T cells, and splenic DC in the absence of the OVA peptide.

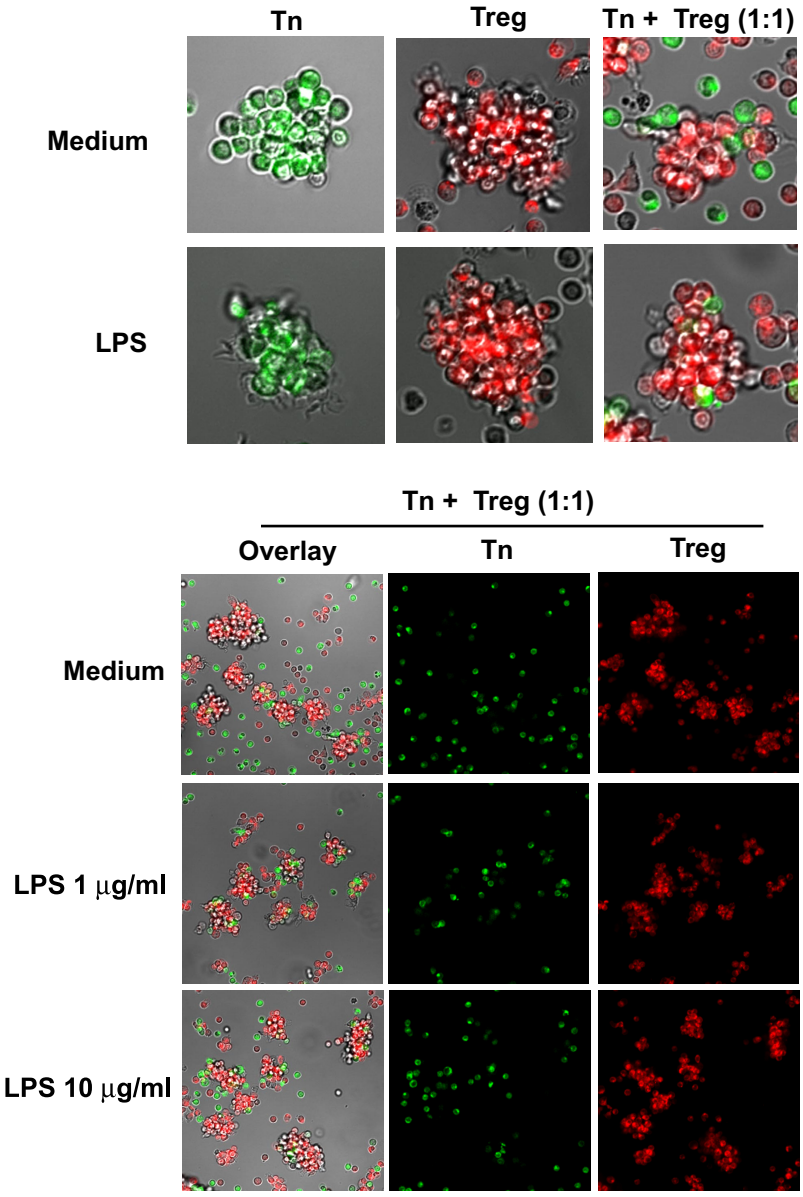

Fig. S2. Preferential Treg aggregation on splenic DCs in presence of LPS. Green-dye labeled Tn cells and red-dye labeled Tregs from DO11.10 mice were cultured for 12 hours with 1 μg/ml or 10 μg/ml LPS, or without LPS, and stimulated with 1 μM $OVA_{323–339}$. Results represent three independent experiments.

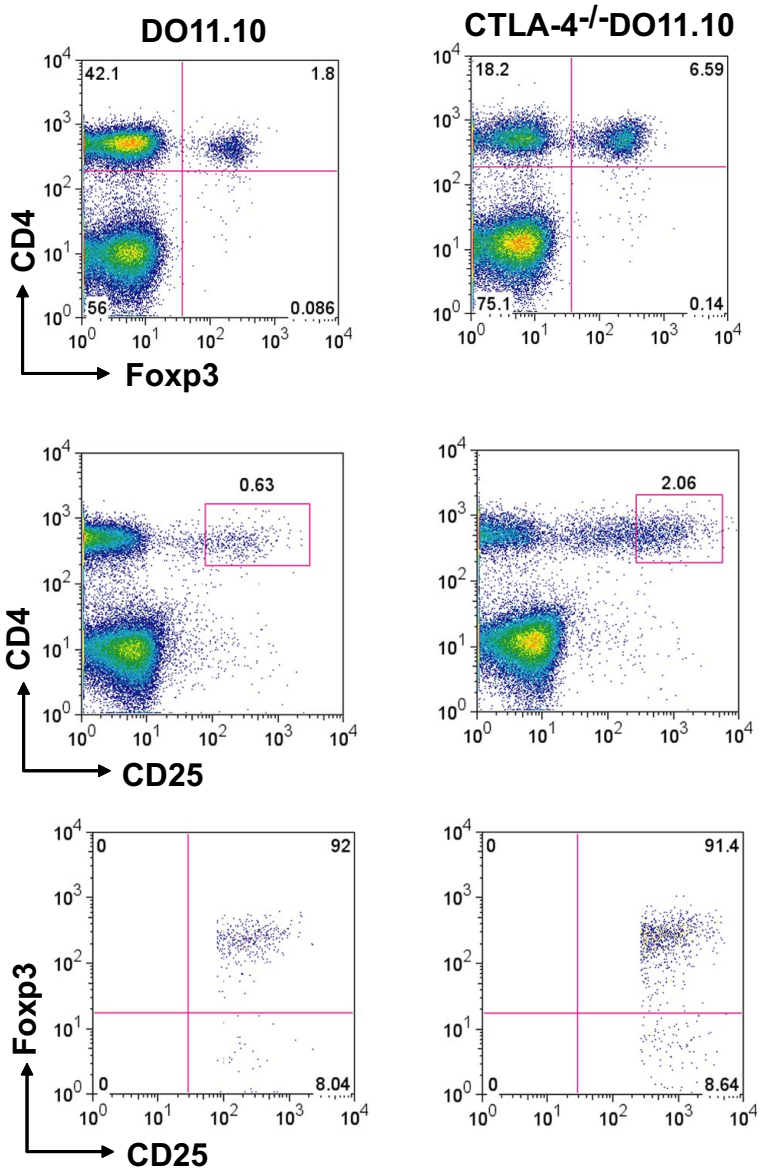

Fig. S3. Purity of Foxp3^{+} Tregs. Spleen and lymph node cells from DO11.10 or CTLA-4$^{-/-}$ DO11.10 mice were stained for intracellular Foxp3 or cell surface CD25 (*Top* and *Middle*). Treg cells prepared as CD25^{high} cells (rectangles, *Middle*) were stained for Foxp3 (*Bottom*), showing a comparable purity of Foxp3^{+} cells from these mice. Anti-Foxp3 (FJK-16s) was purchased from eBioscience.

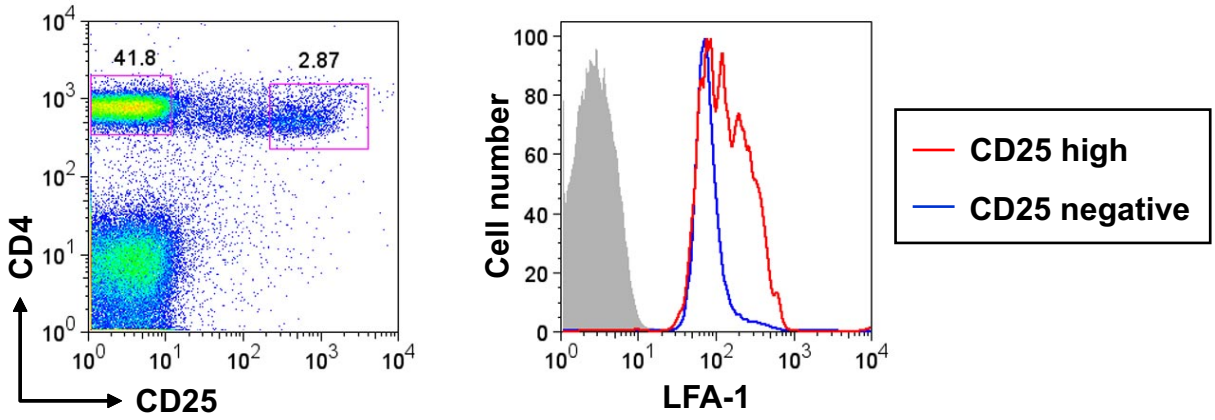

Fig. S4. Higher expression of LFA-1 on $CD4^{+}CD25^{+}$ Tregs in normal mice. Spleen and Lymph node cells from BALB/c mice were stained with FITC anti-CD11a, PE anti-CD25, and APC anti-CD4.

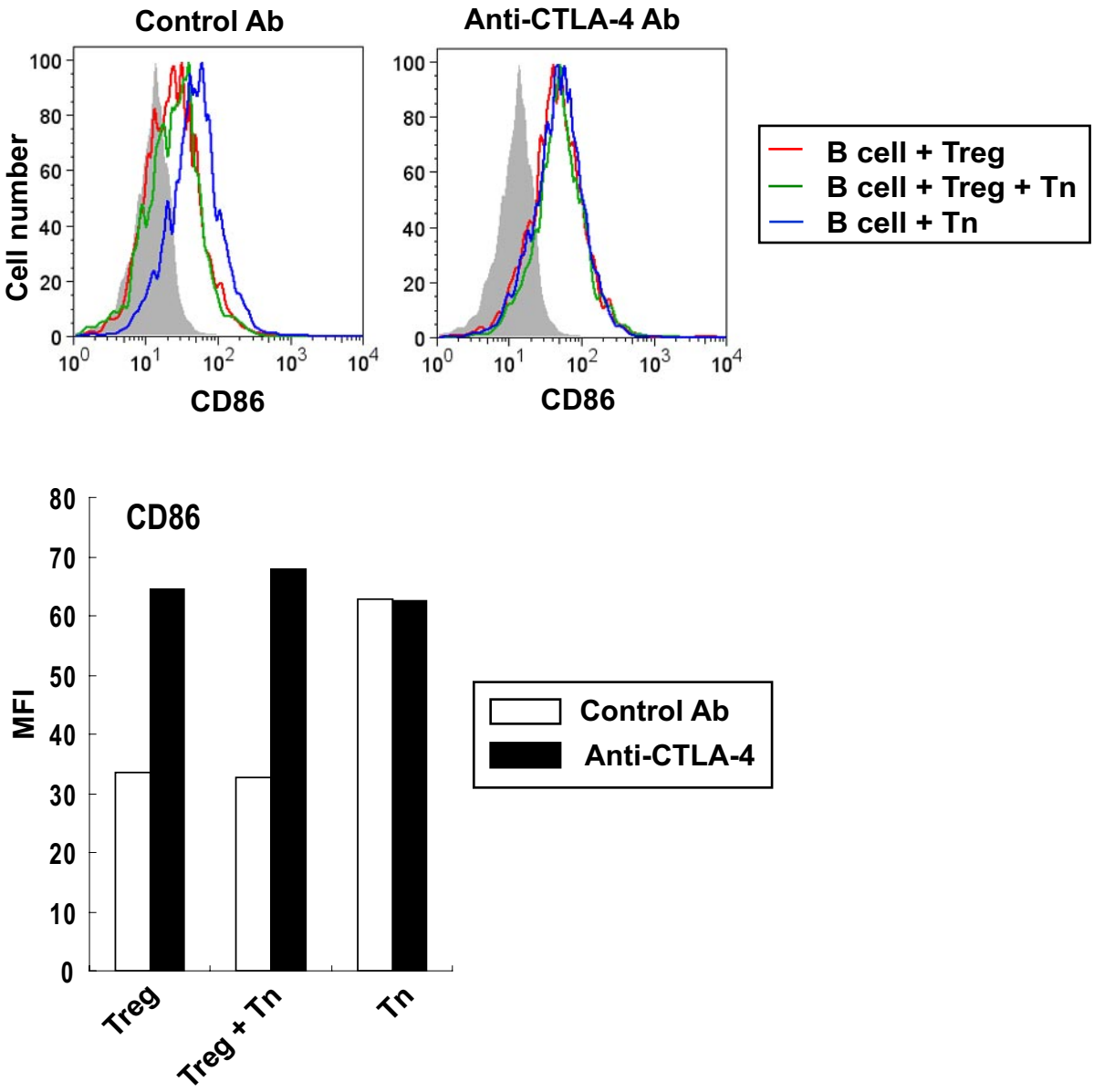

Fig. S5. Down-regulation of CD86 expression on B cells by Treg cells. Splenic B cells from BALB/c mice were cultured for 2 days with Tn or Treg cells from DO11.10 mice, or a mix of the two populations at 1:1 ratio, in the presence of 1 μM $OVA_{323–339}$ and anti-CTLA-4 mAb or control Ab; then CD86 expression on B cells was assessed. A representative staining (*Upper*) and MFI of each B cell population (*Lower*) are shown.

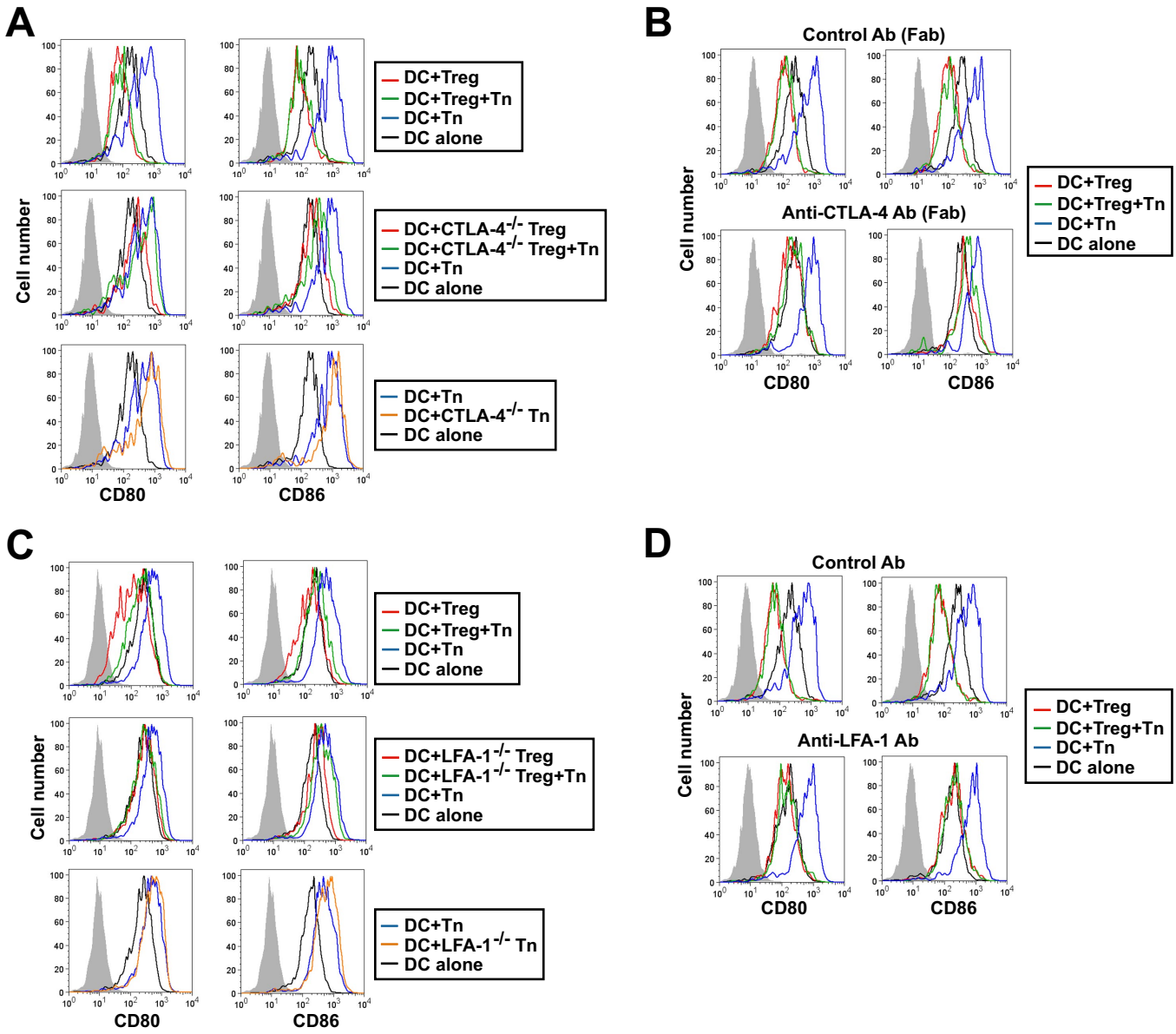

Fig. S6. CTLA-4- and LFA-1-dependent down-regulation of CD80 and CD86 expression on splenic DCs. Splenic DCs from BALB/c mice were cultured 2 days with Tn or Treg cells, or a mix of both populations at a 1:1 ratio, and then CD80 and CD86 expression on splenic DCs was determined. (*A*) Tn and Treg cells from DO11.10 or CTLA-$4^{-/-}$ DO11.10 mice were cultured with 1 μM $OVA_{323–339}$. (*B*) Tn and Treg cells from DO11.10 mice were cultured with 100 μg/ml anti-CTLA-4 mAb (Fab) or control Ab (Fab). (*C*) Tn and Treg cells from wild type or LFA-$1^{-/-}$ mice were cultured with 0.1 μg/ml anti-CD3 mAb. (*D*) Tn and Treg cells from DO11.10 mice were cultured with 2 μg/ml anti-LFA-1 mAb or control Ab. Results in *A–D* represent three independent experiments.

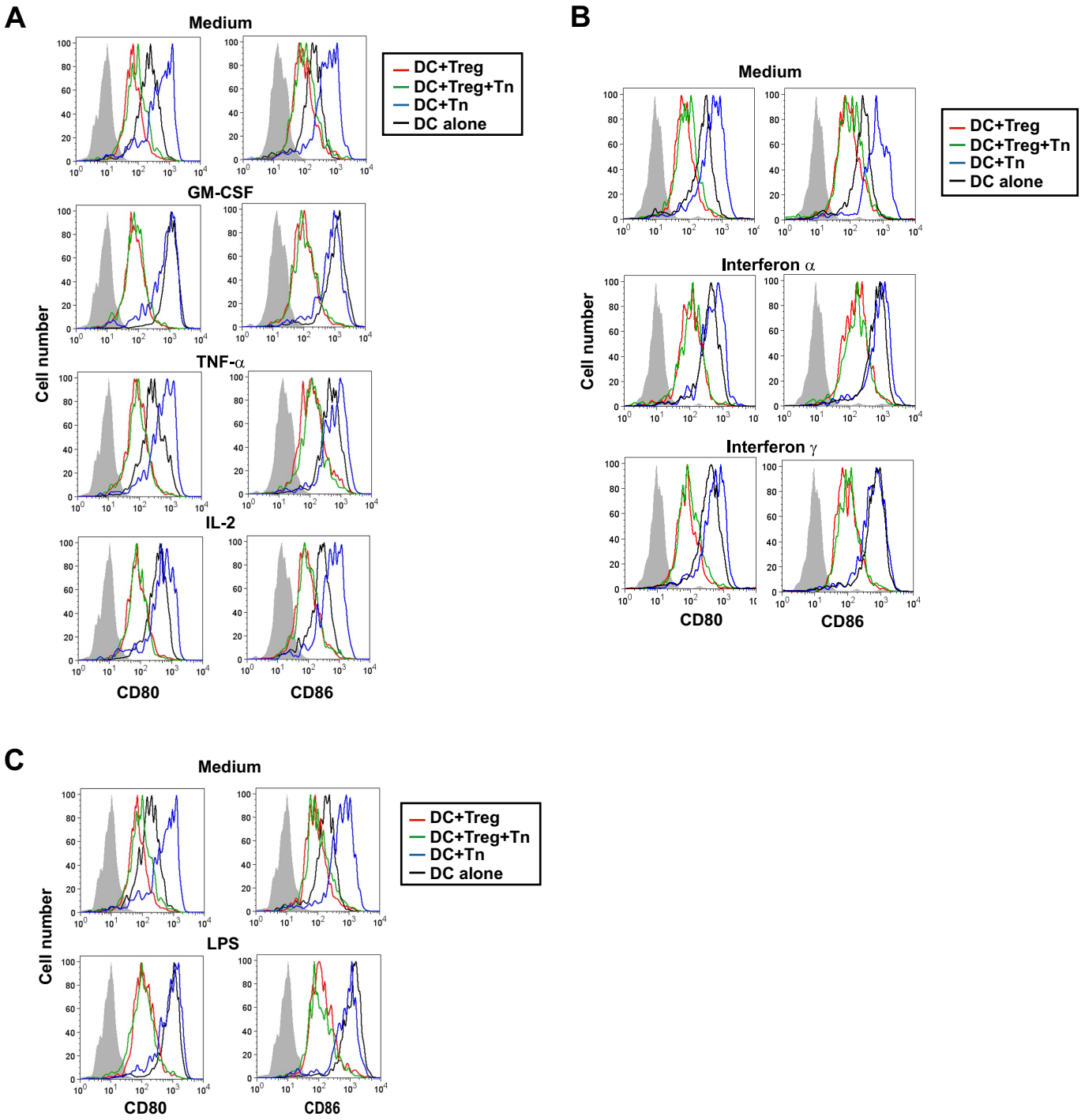

Fig. S7. The effects of cytokines and LPS on CD80/86 expression of Treg-cocultured splenic DCs. CD80 and CD86 expression on BALB/c splenic DCs was determined after coculture with Tn or Treg cells purified from DO11.10 mice, or with two populations mixed at a 1:1 ratio. The cells were stimulated with 1 μM $OVA_{323-339}$ in the presence of GM-CSF (100 ng/ml), TNF-α (20 ng/ml), or IL-2 (200 units/ml) (*A*); IFNα (1,000 units/ml) or IFNγ (100 ng/ml) (*B*); LPS (1 μg/ml) (*C*). Results in *A*–*C* represent three independent experiments.

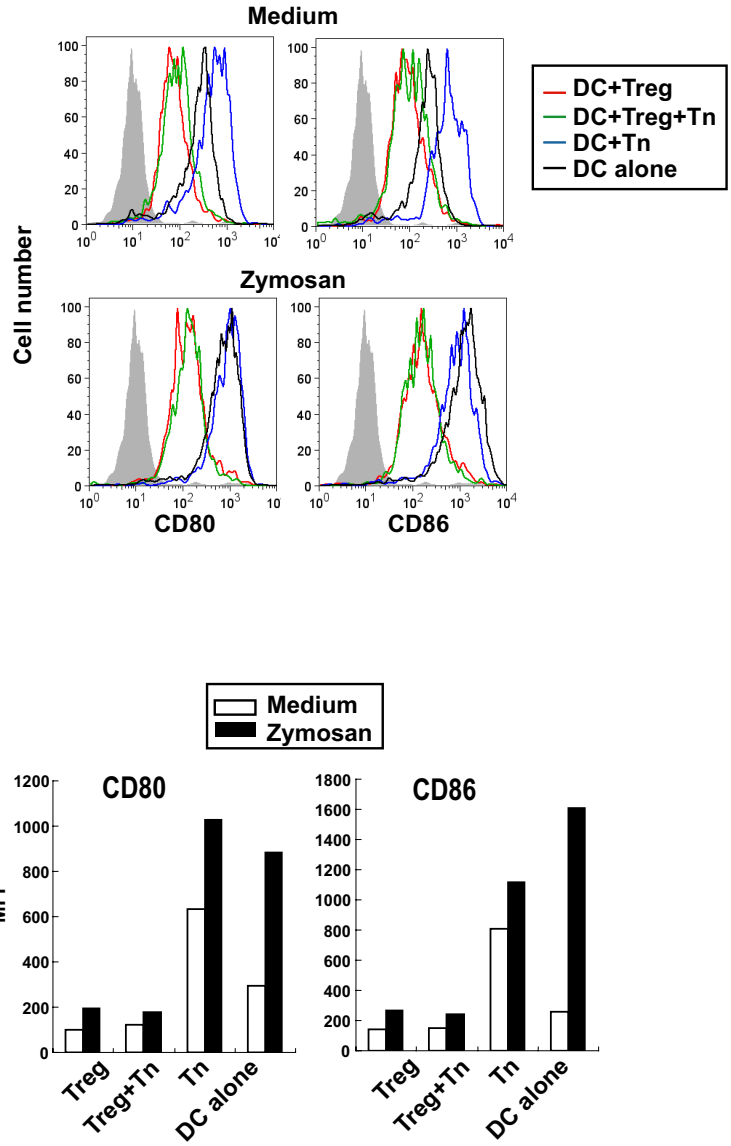

Fig. S8. The effects of Zymosan on CD80/86 expression of Treg-cocultured DCs. CD80 and CD86 expression on BALB/c splenic DCs was determined after coculture with Tn or Treg cells purified from DO11.10 mice, or with two populations mixed at a 1:1 ratio. The cells were stimulated with 1 μM $OVA_{323–339}$ in the presence of Zymosan (100 μg/ml). Results represent three independent experiments.

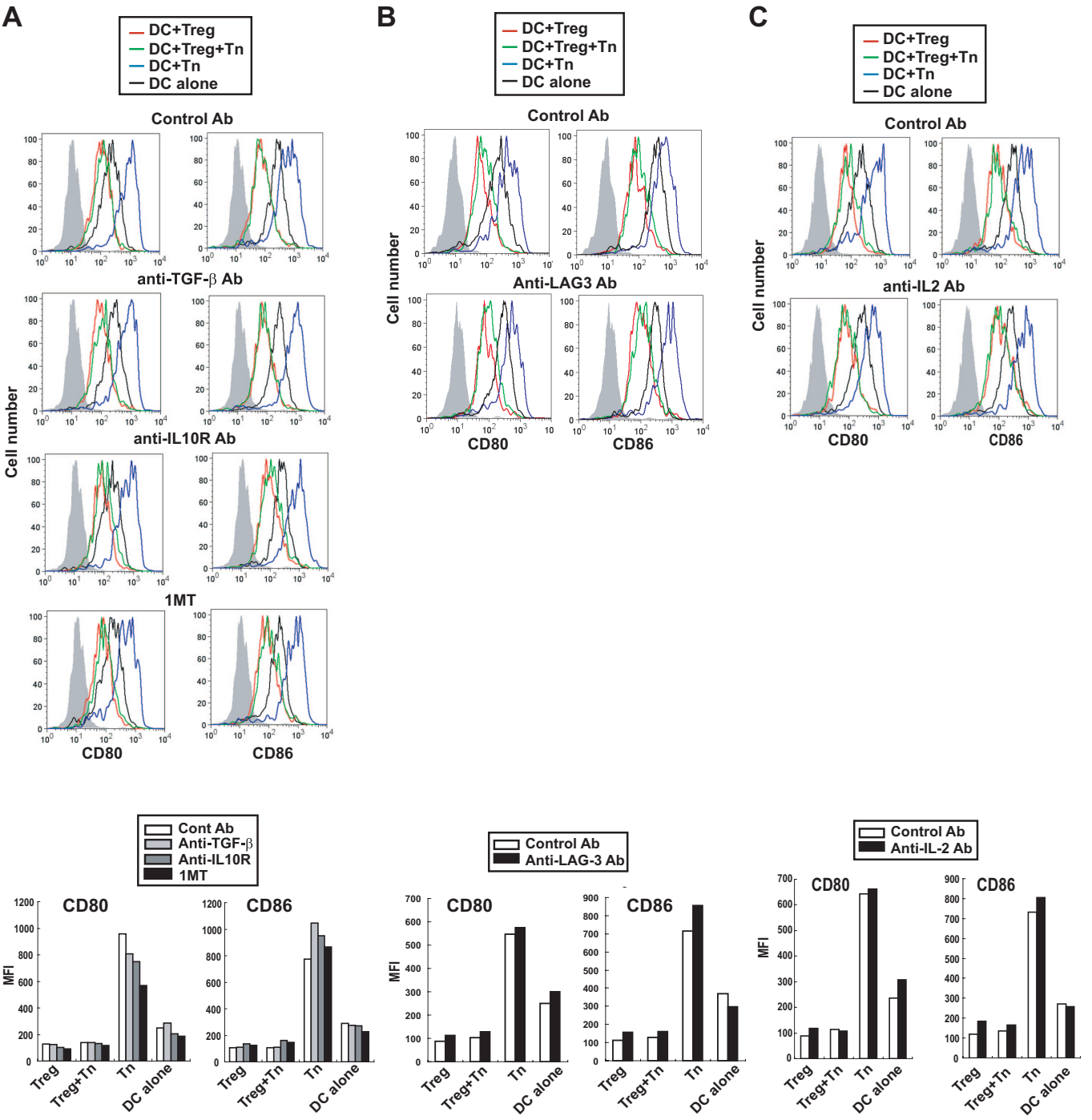

Fig. S9. The effects of biological reagents on CD80/86 expression of Treg-cocultured splenic DCs. CD80 and CD86 expression on BALB/c splenic DCs was determined after coculture with Tn or Treg cells purified from DO11.10 mice, or with two populations mixed at a 1:1 ratio. The cells were stimulated with 1 μM $OVA_{323–339}$ in the presence of neutralizing anti-TGF-β mAb (50 μg/ml), anti-IL10R mAb (10 μg/ml), or 1MT (100 μM) for inhibition of IDO (*A*); anti-LAG-3 mAb (30 μg/ml) or control Ab (*B*); neutralizing anti-IL-2 mAb (50 μg/ml) or control Ab (*C*). Staining profile and MFI of each experiment are shown. Results in *A*–*C* represent three independent experiments.

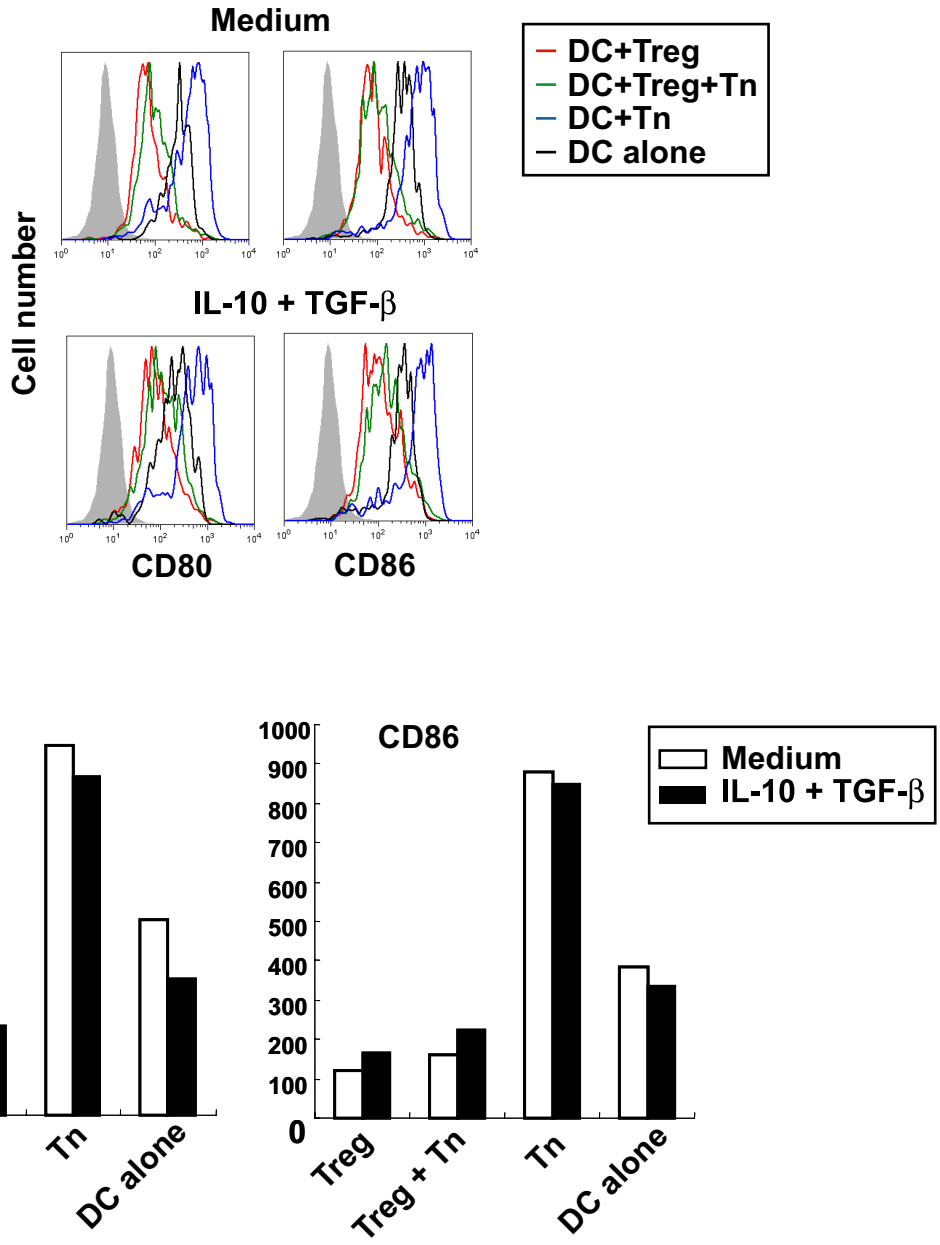

Fig. S10. The effects of TGF-β and IL-10 on CD80/86 expression of Treg-cocultured splenic DCs. CD80 and CD86 expression on BALB/c splenic DCs was determined after coculture with Tn or Treg cells purified from DO11.10 mice, or with two populations mixed at a 1:1 ratio. The cells were stimulated with 1 μM $OVA_{323–339}$ in the presence TGF-β (20 ng/ml) and IL-10 (40 ng/ml). Results represent three independent experiments.

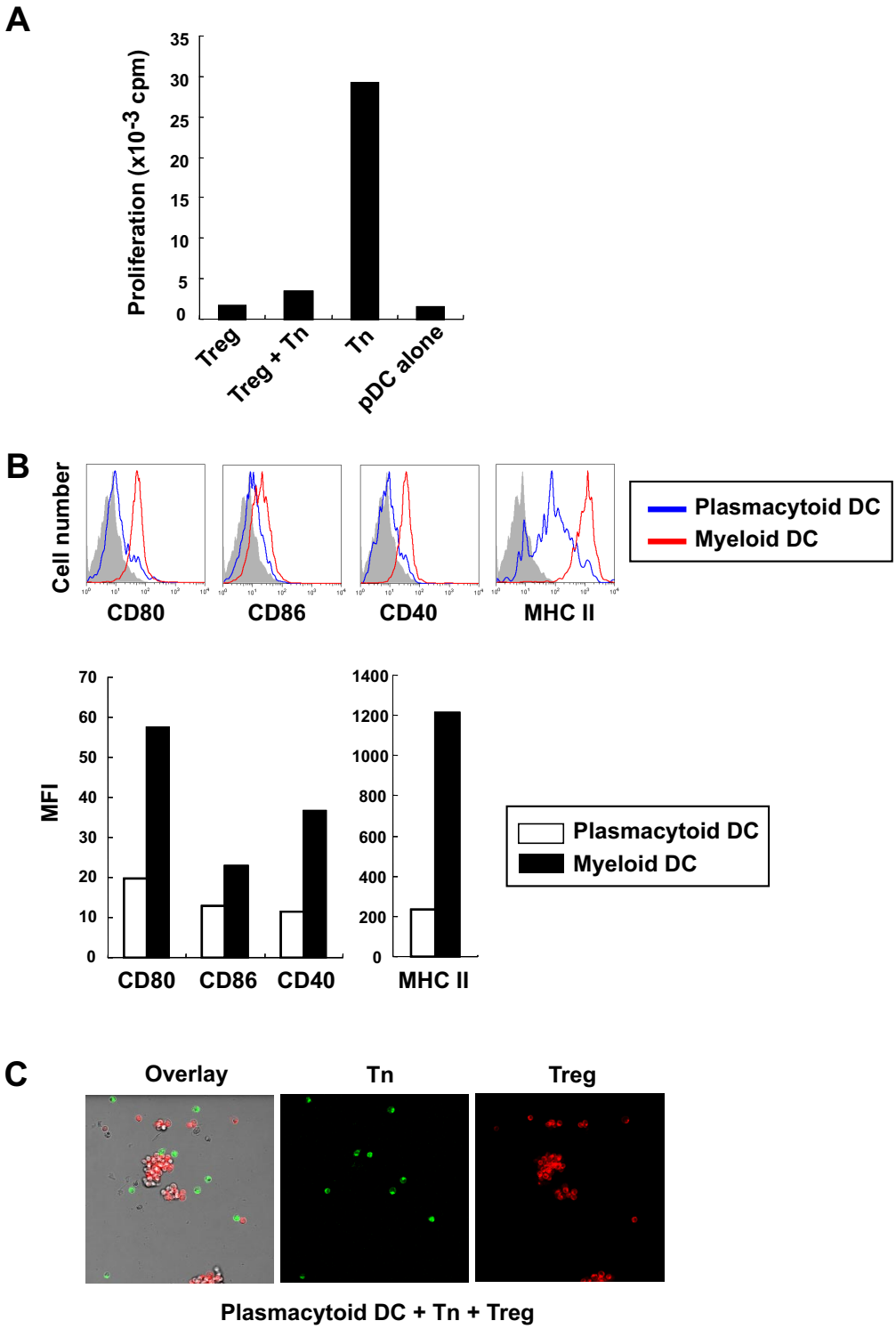

Fig. S11. The effects of Tregs on plasmacytoid DCs. (*A*) DO Tn cells, DO Tregs, or a mix of the two populations at a 1:1 ratio, were cultured in the presence of plasmacytoid DCs (pDCs). The cells were stimulated with 1 μM $OVA_{323–339}$. (*B*) CD80, CD86, CD40, and MHC class II expression on splenic myeloid DCs and pDCs before the culture. (*C*) Preferential Treg aggregation on pDCs. Green-dye labeled Tn cells and red-dye labeled Tregs were cultured for 12 hours with $CD11c^{low}CD11b^{-}B220^{+}$ pDCs in the presence of the $OVA_{323–339}$. pDCs were isolated from spleens by the treatment with Librase Blendzyme II and sorted by cell sorter.

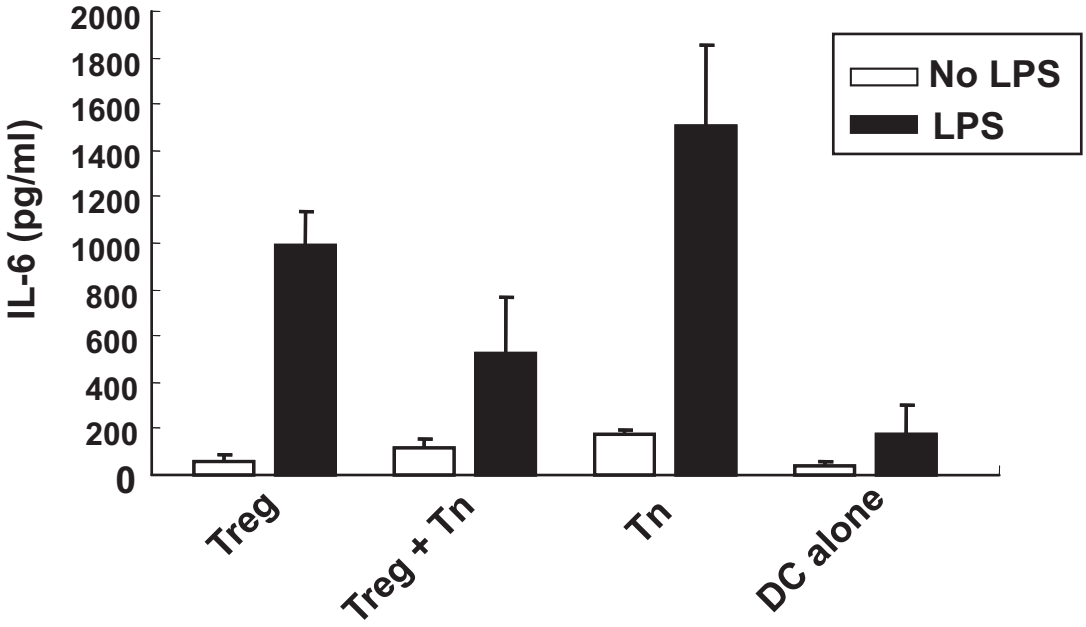

Fig. S12. Production of IL-6 in presence of LPS. The concentration of IL-6 in supernatant of the culture of DO Tn cells, DO Tregs, or a mix of the two populations in the presence of splenic DCs was measured by ELISA using Mouse IL-6 Ready-SET-Go! (eBioscience), with a detection limit of 4 pg/ml. The cells were stimulated for 2 days with 1 μM $OVA_{323–339}$ in the presence of LPS (1 μg/ml).