Supporting Information

Valmori et al. 10.1073/pnas.1008247107

SI Materials and Methods

Samples, Cell Purification, and ex Vivo Cell Sorting. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by density gradient sedimentation using LSM 1077 lymphocyte separation medium (PAA Laboratories). $CD4^+$ T cells were enriched by positive selection by magnetic cell sorting (Miltenyi Biotec). For ex vivo flow cytometry cell sorting, enriched $CD4^+$ T cells were stained with mAb specific for CD4, CD8, CD45RA (BD Biosciences), CD25 (Beckman Coulter), and CD127 (eBioscience), and $CD4^+CD8^-CD45RA^+$ lymphocytes were separated into naive $CD25^-CD127^{high}$ conventional $CD4^+$ T cells (N) and naive $CD25^+CD127^{low}$ Treg (NTreg) to high purity (>97%) using a FACSAria (BD Biosciences). Aliquots of sorted populations were stained with anti-FOXP3 mAb (eBioscience) and analyzed by flow cytometry.

Phenotypic Assessment and Flow Cytometry Cell Sorting of Differentiated Cultures. On day 5 or 12 following in vitro stimulation, aliquots of differentiated N and NTreg cultures were stained with fluorochrome-labeled anti-IL-1RI mAb (R&D Systems) and/or anti-CCR6 mAb (BD Biosciences), or unlabeled anti-IL-23R antibodies followed by fluorochrome-labeled goat anti-rabbit Ig antibodies (R&D Systems) and analyzed by flow cytometry. For some experiments, day 12 cultures were stained with anti-CCR6 mAb and sorted into $CCR6^+$ and $CCR6^-$ populations or with anti-IL-1RI and anti-CCR6 mAb and sorted into $IL\text{-}1RI^+CCR6^-$, $IL\text{-}1RI^+CCR6^+$, and $IL\text{-}1RI^-CCR6^+$ populations by flow cytometry.

Assessment of Cytokine Production and RORγt and FOXP3 Expression. In vitro differentiated $CD4^+$ T cells were stimulated with PMA (100 ng/mL, Sigma Aldrich) and ionomycin (1 μg/mL, Sigma Aldrich) for 5 h. Brefeldin A (10 μg/mL, Sigma Aldrich) was added 1 h after the beginning of the incubation. Cells were then fixed, permeabilized, and stained with mAb specific for IL-17A (eBioscience), IL-17F (eBioscience), IL-2 (BD Biosciences), IL-4 (BD Biosciences), IL-10 (BD Biosciences), IL-21 (BD Biosciences), IL-22 (R&D systems), IFN-γ (BD Biosciences), and TNF-α (BD Biosciences), as indicated, and analyzed by flow cytometry. Expression of RORγt and FOXP3 with respect to IL-17 production was also assessed following PMA and ionomycin stimulation, specific mAb staining (eBioscience), and flow cytometry analysis.

Assessment of Suppressor Function. The ability of in vitro differentiated or ex vivo sorted $CD4^+$ T-cell populations to suppress the growth of responder $CD4^+CD25^-$ T cells was assessed by coculture of CFSE- (Invitrogen) labeled responder conventional $CD4^+$ T cells (3×10^4) with or without test populations, in 96-well U-bottom plates, in the presence of 3×10^4 irradiated monocytes and PHA (HA16, 0.5 μg/mL, OXOID). Growth of responder cells was assessed by flow cytometry analysis of CFSE dilution in day 4–6 cultures. The growth (100 − % undivided cells) of the wells with suppressor cells (experimental group) was compared with that of the wells without suppressors (control). The percentage of suppression was determined as follows: 100 − [(growth of experimental group/growth of control) ×100].