Supporting Information

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SI Materials and Methods

CCR4 Inhibition. For experiments in which CCR4 was inhibited, mice were injected i.p. with 1.5 μg of CCR4 antagonist C-021 dihydrochloride (EMD Millipore) in 100 μL of PBS 12 h before apoptotic cell administration. This amount was equivalent to dosing reported for another CCR4-antagonizing compound (AF399/420/18025) that blocks CCR4 function in vivo (1–4). C-021 in vivo administration has only been reported via oral and s.c. injection to our knowledge with doses ranging from 100 mg/kg (oral) to 3 mg/kg s.c (5, 6). Our dosage is substantially below these reported amounts; however, it was determined empirically to inhibit Treg accumulation in the splenic follicle (Fig. 2 *A* and *B*) and thus was used throughout the studies for in vivo inhibition.

Flow Cytometry. To assay for apoptotic cell uptake, 10^7 PKH26-labeled apoptotic cells in 200 μL of PBS was injected i.v. into B6 mice. Two hours after apoptotic cell injection, spleens were collected and injected with 100 U of collagenase (Sigma) in 2 mL PBS and incubated for 30 min at 37 °C in 5 mL PBS containing 400 U/mL of collagenase. From the digest, single-cell suspensions were generated and incubated with MOMA-1 monoclonal antibody (Serotec).

To examine FoxP3$^+$ Treg positioning in the spleen, B6 mice were injected with 10^7 apoptotic syngeneic thymocytes, and 4 h post-injection spleens were collected and stained with anti-CD4 APC (BD Pharmingen) and anti-FoxP3 PE (eBioscience). Intracellular staining for Foxp3 expression was performed with an intracellular staining kit in accordance with the manufacturer's protocol (eBioscience).

For sorting of macrophage and dendritic cell (DC) subsets, CCR4 antagonist (EMD Millipore) pretreated mice were injected with 10^7 apoptotic syngeneic thymocytes, and 4–8 h post-injection, spleens were collected and treated with collagenase as described above. From the digest, single-cell suspensions were generated and incubated with MOMA-1 (Serotec), α-CD8α, α-CD11c, αCD103 (BD Pharmingen), and α-F4/80 (eBioscience). The cells were sorted on a DakoCytomation MoFlo cell sorter into tubes containing RNA protect reagent (Qiagen).

To measure in vivo T-cell proliferation, 2×10^6 5- (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled (Invitrogen) OTII$^+$CD45.2$^+$ T cells were adoptively transferred i.v. into B6.CD45.1 congenic mice via lateral tail-vein injection. Twenty-four hours after OTII transfer, mice were injected with 10^7 apoptotic Act-mOVA thymocytes in 200 μL of PBS i.v. Three days after apoptotic cell transfer, the spleen was collected and proliferation was assessed via flow cytometry. To visualize the adoptively transferred OTII cells, 5×10^6 splenocytes were stained with 2.5 μg α-CD45.2 PE (eBioscience).

For flow cytometric analysis, $\geq 10^5$ (10^6 in the case of adoptive OTII transfer) events were collected on a LSR II flow cytometer, and all results were analyzed with FlowJo software (TreeStar).

Semiquantitative PCR. RNA from sorted cells was purified using RNeasy RNA purification kits (Qiagen), and 250 ng of RNA was reverse-transcribed using a random hexamer cDNA reverse-transcription kit (Clontech). For the PCR, 1 μL of cDNA was amplified with primers previously described (7). PCR was done using iQ SYBR Green Super Mix (Bio-Rad) and on an iQ5 real-time PCR detection system (Bio-Rad), and results were analyzed with the accompanying software according to the manufacturer's instructions.

Chemotaxis Assay. To assay for chemotaxis, the culture supernatants described in *Materials and Methods* were seeded in the lower chamber of the provided 96-well plates. The upper chambers were seeded with MACS column-sorted CD4$^+$CD25$^+$ Tregs, CD103$^+$ CD8$^+$, or CD8^{neg} DCs in a normal culture medium in the presence or absence of CCR4 antagonist (39 nM C-021 dihydrochloride; EMD Millipore), αCCL22 blocking antibody (2 μg/mL; R&D Systems), or isotype control, and the cells were allowed to migrate for 4 h. The plate was then processed and read at 485 nm (excitation)/530 nm (emission). Migration index was determined as per manufacturer's directions.

Immunofluorescence and Immunohistochemistry. To assay for immune deposits, kidneys were embedded in Tissue-Tek OCT compound (Sakura) and snap-frozen. Sections (5 μm) were air-dried, fixed with cold acetone, and stained with a 1:200 dilution in PBS plus 1% normal goat serum (NGS) of FITC-conjugated α-mouse IgG (Sigma-Aldrich).

For immunohistochemistry, spleen was snap-frozen and embedded as described above. For immunofluorescence staining of FoxP3 in the spleen, 5-μm sections were fixed for 10 min in 100% methanol followed by extensive PBS washing. The sections were blocked with PBS containing 1% (wt/vol) nonfat milk (Sigma) and 5% (vol/vol) NGS and stained with 1:100 dilution (vol/vol) of rabbit anti-mouse FoxP3 (Abcam) and 1:50 dilution (vol/vol) of hamster anti-mouse CD11c or CD103 (BioLegend) in blocking buffer. After extensive washing with TBS + 0.1% Tween-20, the sections were stained with a 1:1,000 dilution of AF488-labeled goat anti-rabbit IgG (Jackson ImmunoResearch) and AF568-labeled donkey anti-hamster IgG (Jackson ImmunoResearch). Sections were mounted with Prolong Gold anti-fade with DAPI (Invitrogen). In quantifying Treg and DC accumulation, at least 20 follicles were examined per spleen. Fluorescent images were captured using a Zeiss LSM 510 Meta confocal microscope equipped with 405-, 488-, 561-, and 633-nm lasers.

Skin Transplantation. Before skin transplantation, female recipient (B6) mice were injected i.p. with 1.5 μg of CCR4 antagonist as described above, or B6.CD169^{+DTR} mice were depleted of metallophillic macrophages as described previously (7). One day later, the mice received 10^7 apoptotic thymocytes (i.v.). After 1 wk (7 d), tail skin from donor male mice (~1 cm^2) was placed onto the left thoracic flank region of female mice as previously described (8). Bandages were removed 7 d after engraftment, and allografts were observed for 50 d. Grafts were scored as rejected when ulceration and/or necrosis was evident in >80% of the graft.

Assays for Autoantibodies. Briefly, Immulon 2 plates (Dynatech) precoated with BSA were coated with 50 μg/mL calf thymus dsDNA (Sigma). To assay for serum autoantibody levels, 50 μL of whole blood was collected from tail vein, and the serum was separated using blood collection microtubes according to the manufacturer's directions (Sarstedt). The serum was diluted 1:200 and assayed for autoantigen reactivity against the plates described above by incubation for 2 h at room temperature. Bound IgG was detected using a goat HRP anti-mouse IgG and IgG3 detection antibody (Bethyl Laboratories) and visualized at 450 nm using TMB substrate (KPL) according to the manufacturer's directions.

Image and Statistical Analysis. Image analysis was done using National Institutes of Health ImageJ software unless otherwise indicated. Means, SDs, and unpaired Student *t* test results were used to analyze the data. When comparing two groups, a $P \leq 0.05$ was considered to be significant. Allograft survival data were analyzed with Kaplan–Meier survival plots followed by the log-rank test.

1. Yokoyama K, et al. (2008) Discovery of potent CCR4 antagonists: Synthesis and structure-activity relationship study of 2,4-diaminoquinazolines. *Bioorg Med Chem* 16(14):7021–7032.
2. Yokoyama K, et al. (2008) Potent CCR4 antagonists: Synthesis, evaluation, and docking study of 2,4-diaminoquinazolines. *Bioorg Med Chem* 16(17):7968–7974.
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4. Pere H, et al. (2011) A CCR4 antagonist combined with vaccines induces antigen-specific CD8+ T cells and tumor immunity against self antigens. *Blood* 118(18):4853–4862.
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6. Chow Z, Mueller SN, Deane JA, Hickey MJ (2013) Dermal regulatory T cells display distinct migratory behavior that is modulated during adaptive and innate inflammation. *J Immunol* 191(6):3049–3056.
7. Ravishankar B, et al. (2012) Tolerance to apoptotic cells is regulated by indoleamine 2,3-dioxygenase. *Proc Natl Acad Sci USA* 109(10):3909–3914.
8. Dunn LA, et al. (1997) Presentation of the HPV16E7 protein by skin grafts is insufficient to allow graft rejection in an E7-primed animal. *Virology* 235(1):94–103.

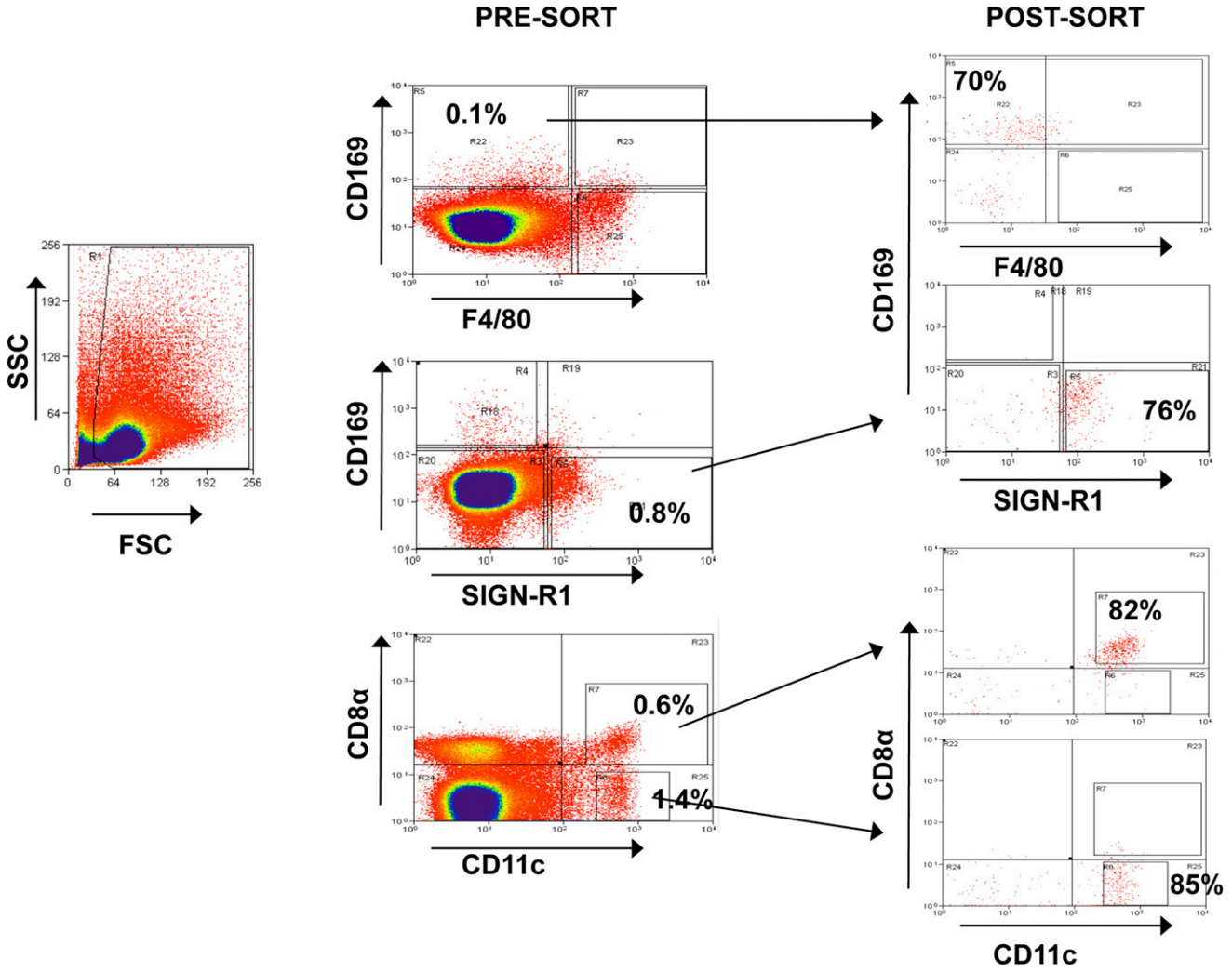

Fig. S1. FACS sorting scheme for splenic MΦs and DCs. Representative dot plots for pre- and postsort of splenic cell populations indicated. Spleens from 3–5 mice per group were pooled for each sort. Experiment was repeated multiple times with similar results. FSC, forward scatter, SSC, side scatter.

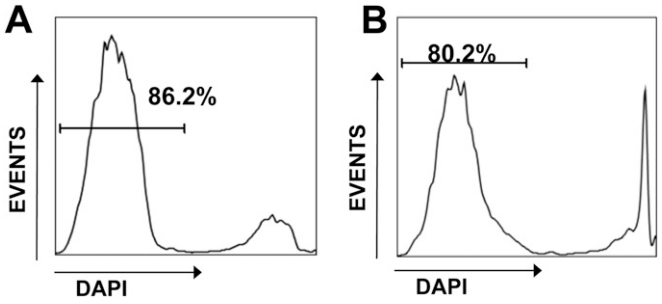

Fig. S2. CD169+ MΦ viability in culture. Sorted CD169^{+} macrophages were stained with DAPI to determine cell viability immediately after FACS sorting (*A*) and after 24 h coculture with apoptotic cells (*B*) as described in *Materials and Methods*. Histograms show DAPI fluorescence for CD169^{+} cells. Experiment was repeated four times with similar results.

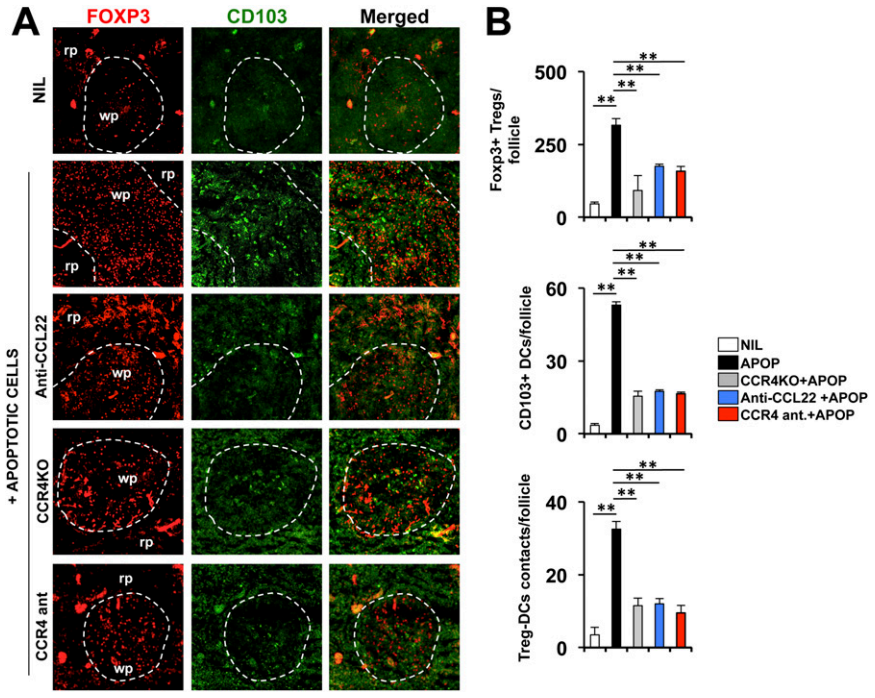

Fig. S3. CCL22 and CCR4 are required for apoptotic cell-driven recruitment of FoxP3^{+} Tregs and CD103^{+} DCs into the splenic follicle. B6 or CCR4KO mice were pretreated as indicated with CCR4 antagonist or CCL22 neutralizing antibody as described in *Materials and Methods* 6 h before injection of 10^{7} apoptotic thymocytes i.v. Four hours after apoptotic cell administration the spleen was collected for analysis. (*A*) Representative immunofluorescence staining of splenic sections to determine localization of CD103^{+} DCs (green) and FoxP3^{+} Tregs (red) after apoptotic cell challenge. Rp, red pulp; wp, white pulp. (*B*) Image analysis of splenic sections from mice in *A* for semiquantitative analysis of follicular CD103+ DC or FoxP3^{+} Treg accumulation and Treg/DC interactions after apoptotic cell challenge. Distance between Tregs and DCs considered contacts was 0.02 μm or less. Distance was quantified by Applied Precision Software (Softworx) on images captured as described in *Materials and Methods*. Images in *A* are representative for five or more mice and are 200× magnification. $^{**}P^{val} < 0.01$ as determined by Student *t* test. Experiments were repeated twice with similar results.

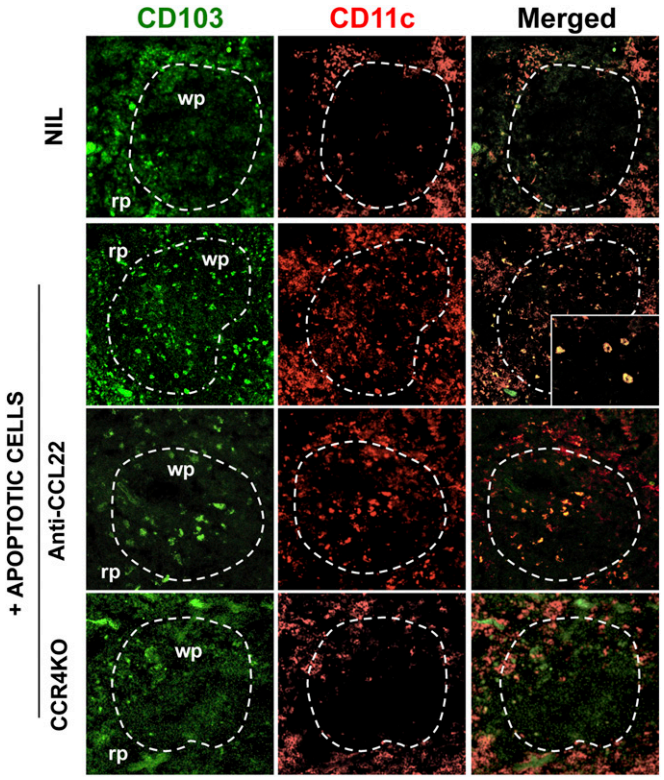

Fig. S4. Apoptotic cell-driven CD11c^{+}CD103^{+} DC recruitment is dependent on CCL22 and CCR4. B6 or CCR4KO mice were pretreated as indicated with CCL22 neutralizing antibody as described in *SI Materials and Methods* 6 h before injection of 10^{7} apoptotic thymocytes i.v. Four hours after apoptotic cell administration the spleen was collected for analysis. Images are representative immunofluorescence staining of splenic sections to determine localization of CD103^{+} (green) and CD11c^{+} (red) DCs after apoptotic cell challenge. Rp, red pulp; wp, white pulp. Images are representative for five or more mice and are 200× magnification. Experiment was repeated two times with similar results.

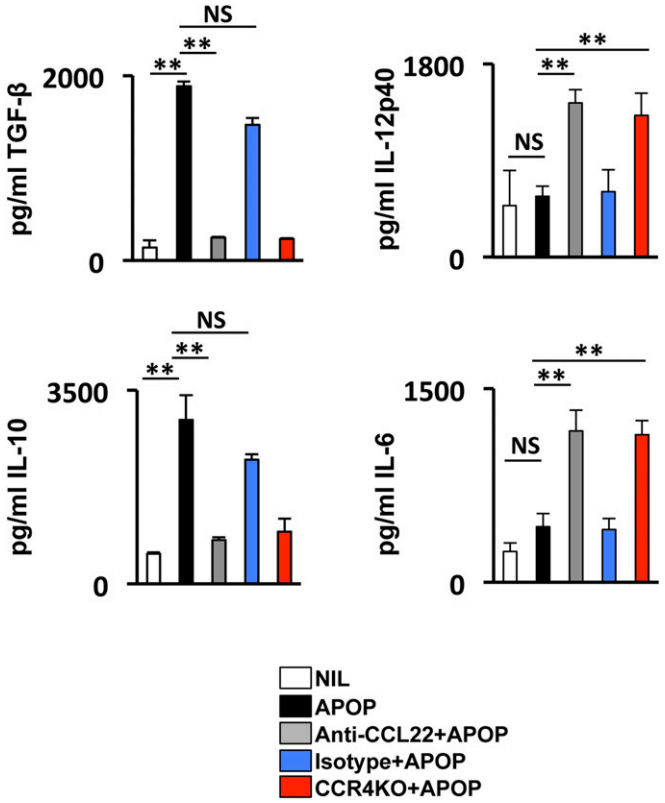

Fig. S5. CCR4 function is required for the apoptotic cell-induced regulatory cytokine response in the spleen. CCR4KO or B6 mice were injected as indicated i.p. with CCL22 neutralizing or control IgG antagonist as described in *SI Materials and Methods*. At 4 h later, the mice were challenged with 10^7 apoptotic syngeneic thymocytes i.v., and 18 h after apoptotic cell administration spleens were collected and cytokine protein concentrations were determined on whole-spleen lysate by ELISA. Bars represent mean value for five mice ± SD. $^{**}P^{val} < 0.01$ as determined by Student *t* test. Experiments were repeated three times with similar results. NS, not significant.

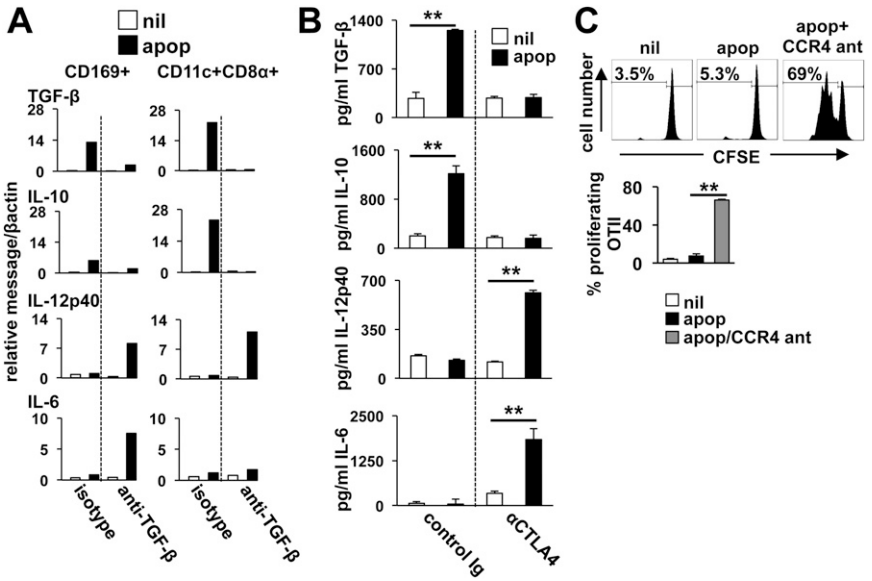

Fig. S6. TGF-β and CTLA4 are required for regulation of innate and adaptive responses to apoptotic cell antigens. (*A*) B6 mice were injected with 150 μg of αTGF-β neutralizing antibody (clone 1D11) or isotype controls i.p., and 8 h later injected with apoptotic cells. At 4 h postinjection, splenic $CD169^+$ macrophages and $CD8\alpha^+CD11c^+$ DCs were purified by FACS, and relative message levels for the cytokine species indicated were determined by sqPCR. (*B*) Mice were injected with 100 μg of αCTLA4 blocking monoclonal antibody (clone 9H10) 24 h before i.v. injection of 10^7 apoptotic cells. At 18 h after apoptotic cell treatment, spleens were collected and splenic lysates were measured for the cytokines indicated by ELISA. For *A*, bars represent the value for pooled samples from three mice. For *B*, bars represent mean value for 5 mice ± SD $^{**}P^{val} > 0.01$ as determined by Student *t* test. Experiments were repeated three times with similar results. (*C*) $CD45.1^+$ B6 mice received 2.5×10^6 MACS-purified CFSE-labeled $CD45.2^+CD4^+$ OTII T cells i.v. followed by challenge with 10^7 OVA^+ apoptotic cells with or without pretreatment with CCR4 antagonist as indicated. Three days later, spleens were collected and CFSE intensity in the $CD4^+CD45.2^+$ T cells and the total number of splenic $CD4^+CD45.2^+$ T cells was determined by flow cytometry. For all experiments, bars represent mean values for triplicate wells ± SD. $^{**}P^{val} < 0.01$ as determined by Student *t* test. Each experiment was repeated multiple times with similar results.