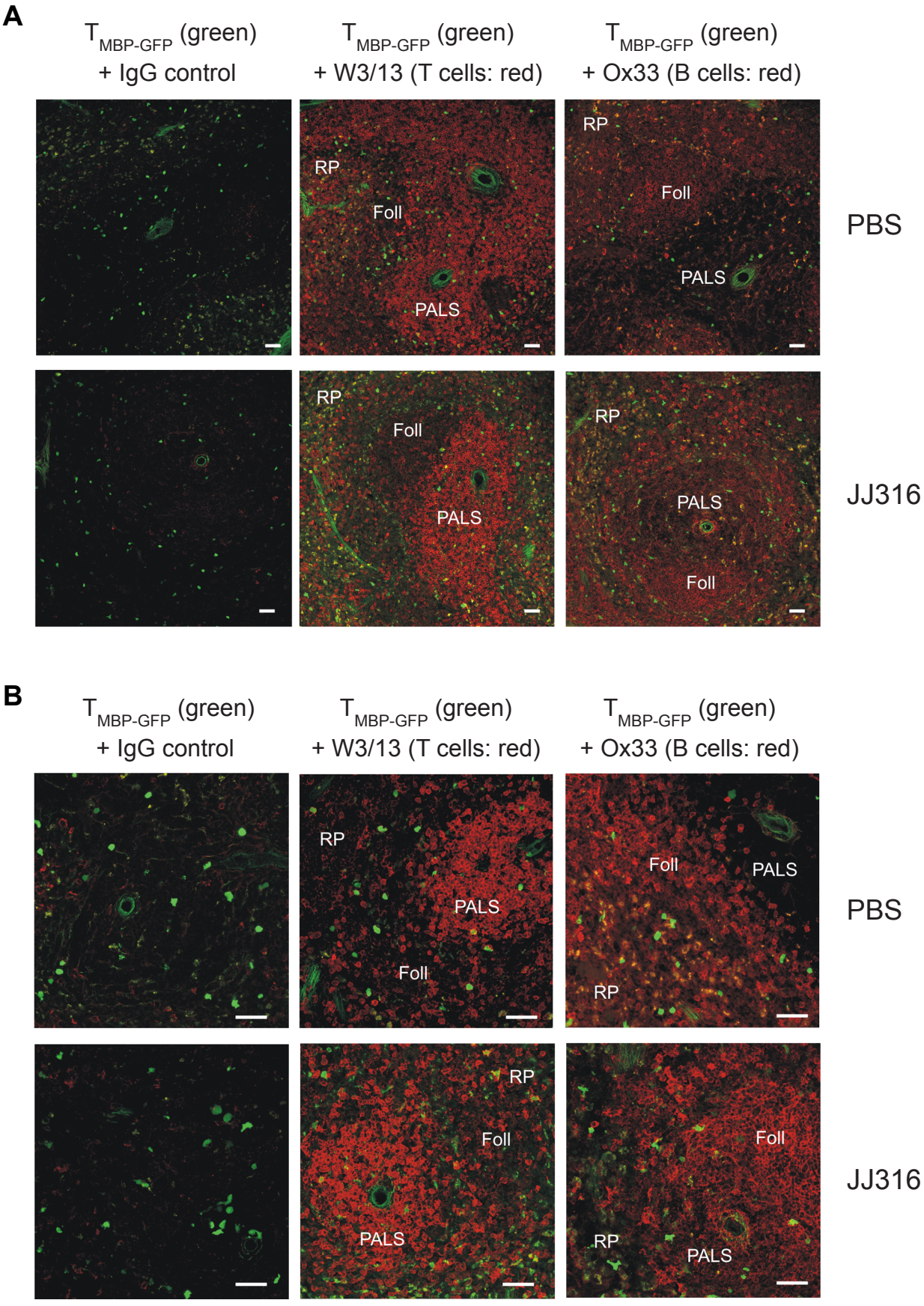
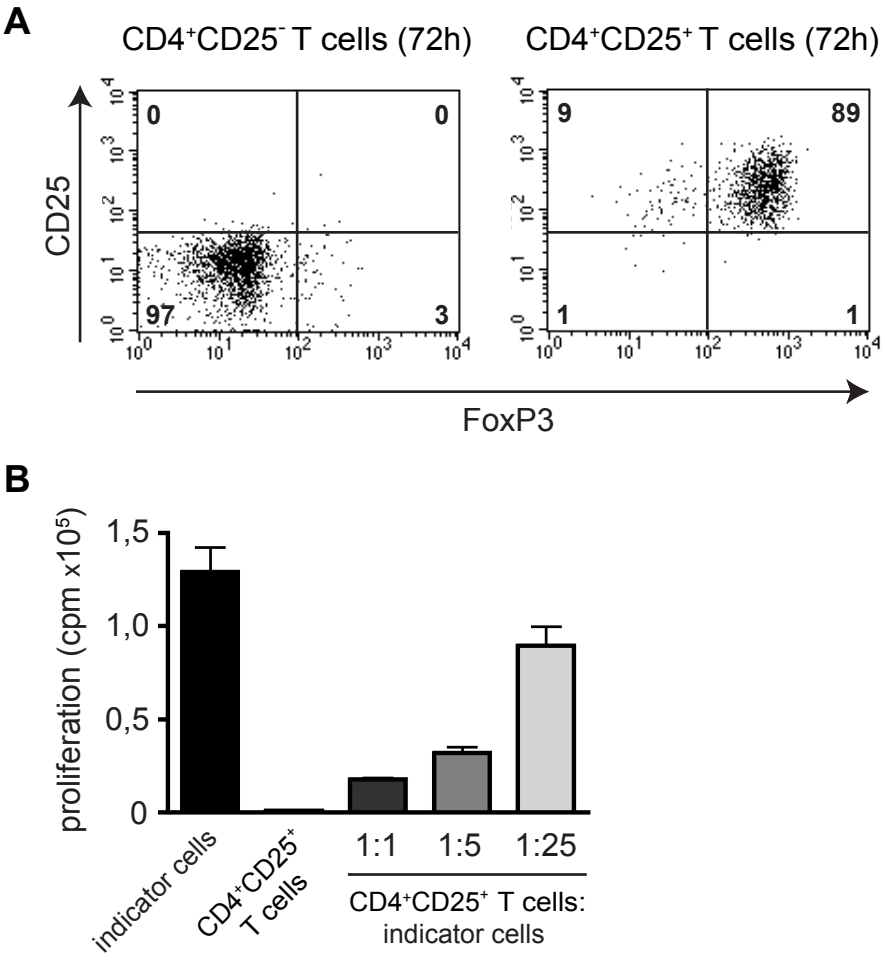
Supplementary Material

CD28 superagonistic antibodies elicit two functionally distinct waves of T cell activation (Müller et al.)



Supplementary Figure 1; Müller et al.



Supplementary Figure 2; Müller et al.

Supplementary Figure legends

Suppl. Fig. 1: Adoptively transferred effector T cells distribute throughout the red pulp and the T cell areas of the spleen. $T_{MBP\text{-}GFP}$ cells were visualized within the spleen 60 hours after adoptive transfer and one hour after infusion of 1.0 mg JJ316 or PBS. $T_{MBP\text{-}GFP}$ cells (green fluorescence) are localized in the red pulp (RP) and the T cell areas of the white pulp (periarteriolar lymphocyte sheaths, PALS). Fewer cells are found within the B cell follicles (Foll). The cell distribution does not substantially change after JJ316 treatment. Sections were additionally stained with mouse IgG (isotype control, left panel), W3/13 to identify T cell areas (red fluorescence, middle panel) or Ox33 to identify B cell areas (red fluorescence, right panel). **(A)** 20x objective; **(B)** 40x oil objective. Magnification bars: 50 μm.

Suppl. Fig. 2: $CD4^+CD25^+$ T cells isolated 72 hours after JJ316 infusion coexpress FoxP3 and suppress T cell proliferation. (A) Rats were injected with 1.0 mg JJ316 and 72 hours later $CD4^+CD25^-$ as well as $CD4^+CD25^+$ T cells were purified by magentic cell sorting followed by flow cytometric analysis for CD25 and FoxP3 expression. The relative percentages of each cell population are depicted. **(B)** The capacity of the $CD4^+CD25^+$ T cells to inhibit the proliferation of conventional T lymphocytes was tested in an in vitro suppression assay based on the incorporation of [^{3}H]-thymidine into DNA. The proliferation of indicator T cells, $CD4^+CD25^+$ T cells and of indicator cells after adding decreasing numbers of $CD4^+CD25^+$ T cells is depicted.

Supplementary Methods

Immunohistochemistry and -fluorescence of tissue sections

Animals having received 5 x 10^6 effector $T_{MBP\text{-}GFP}$ cells by adoptive transfer were perfused with 4% PFA. Subsequently, the spleen was prepared and postfixed in 4% PFA overnight. The frozen organs were cut into 20 µm sections and stained with Ox33 (monoclonal anti-ratCD45RA antibody, specific for B cells, 1:500) or W3/13 (monoclonal anti-ratCD43 antibody, specific for T cells, 1:500) overnight at 4°C (BD Biosciences). The control antibody (MOPC31, mouse IgG) was purchased from Sigma. Cy3-labeled anti-mouse antiserum (Dianova) was used as a secondary antibody. Fluorescence analysis was performed using a confocal laser scanning microscope (Leica).

In vitro suppression assay

$CD4^+CD25^-$ indicator T cells purified from naïve rats ($5x10^5$ cells/ml) were cultured for 72 h together with irradiated spleen cells serving as APCs ($5x10^5$ cells/ml) in the presence of Concanavalin A (2 µg/ml, Sigma) in RPMI-1640 medium supplemented with 10 % FCS and 1 % Pen/Strep using 96-well flat bottom plates. $CD4^+CD25^+$ *bona fide* T_{reg} cells purified from JJ316 treated rats were added to the indicator cell cultures at different amounts (0, $5x10^5$, $1x10^5$, $2x10^4$ cells). As a control, $5x10^5$ $CD4^+CD25^+$ bona fide T_{reg} cells were cultured in the absence of indicator cells. Proliferation was assessed by measuring [^{3}H]-thymidine (Amersham Biosciences) incorporation during the final 16 h of the culturing period. The DNA of [^{3}H]-thymidine-pulsed cells was harvested onto fiberglass filters and the radioactive content was quantitated using a ß-scintillation counter.