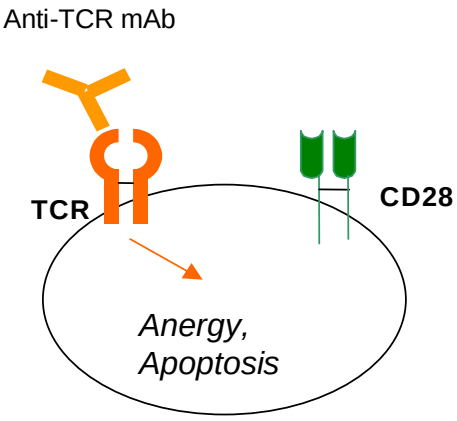
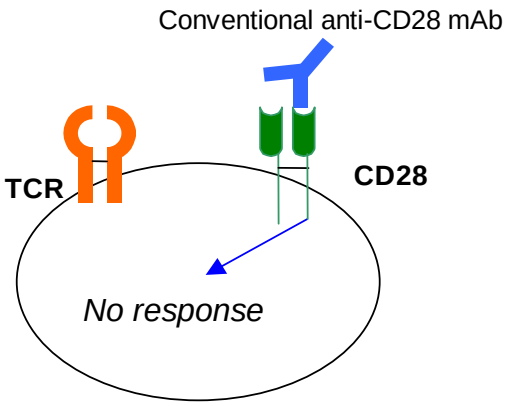
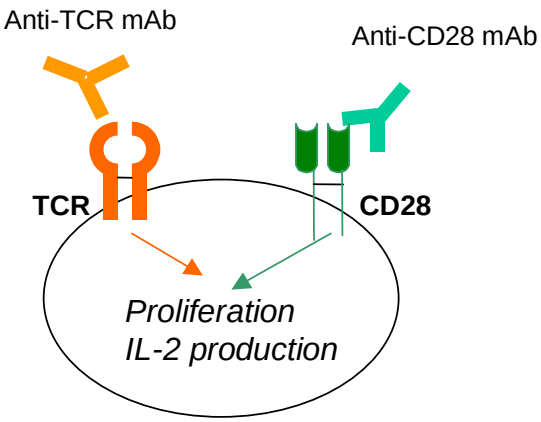
I. Ligation of TCR alone



II. Ligation of CD28 with conventional anti-CD28 mAb



III. Ligation of TCR and CD28 (costimulation)



IV. Ligation of CD28 with CD28 superagonist

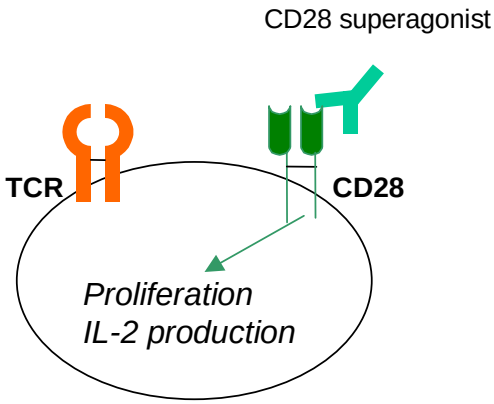


Figure 1. T cell responses upon ligation of TCR or/and CD28 with monoclonal antibodies.

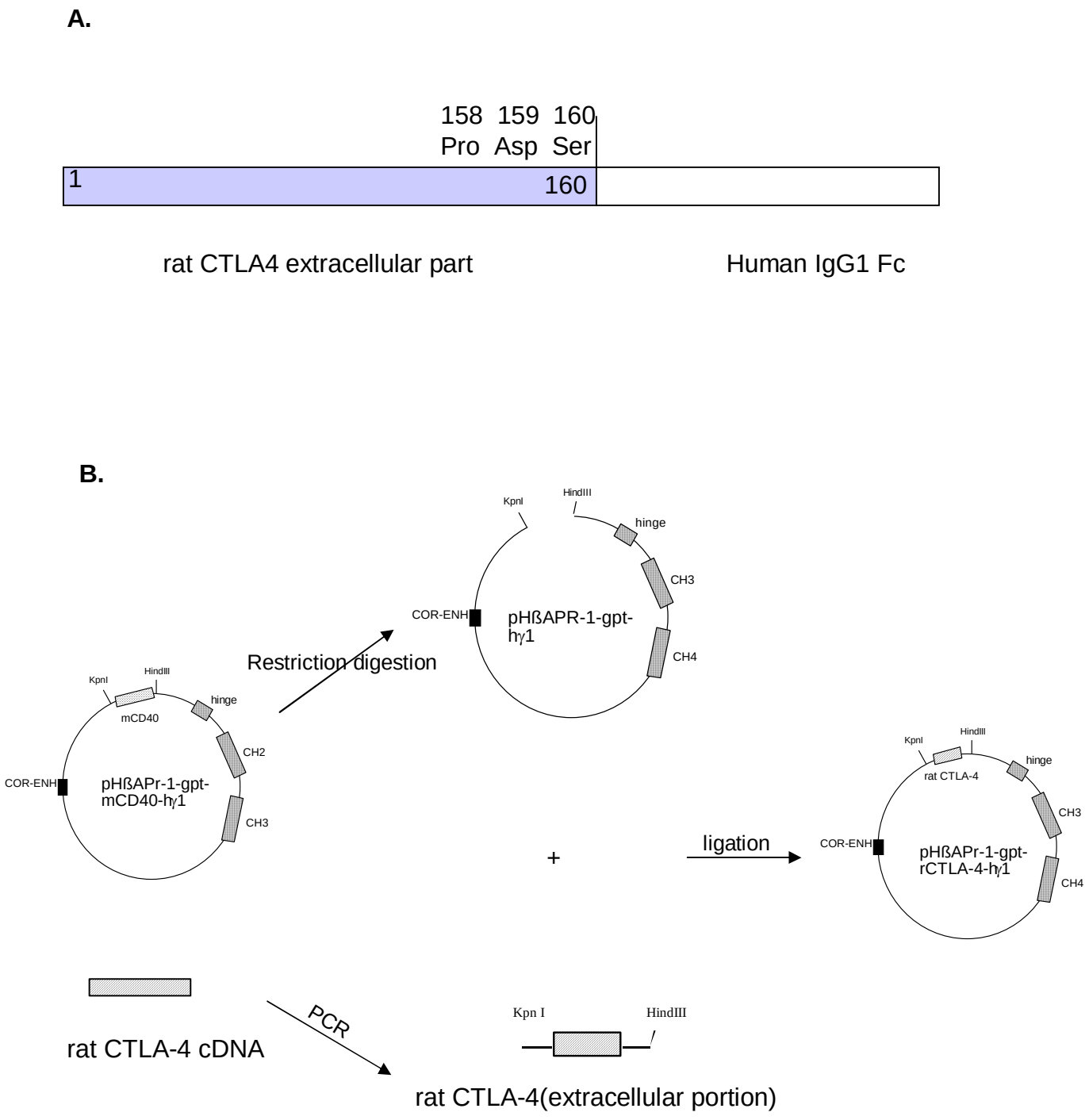
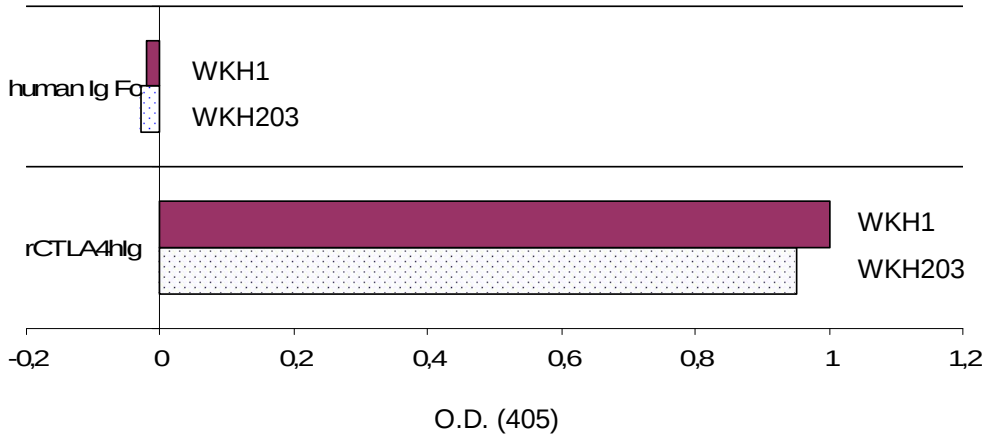


Figure 2. Construction of an expression vector of rCTLA-4hIg fusion protein.

A. ELISA



B. Western blot

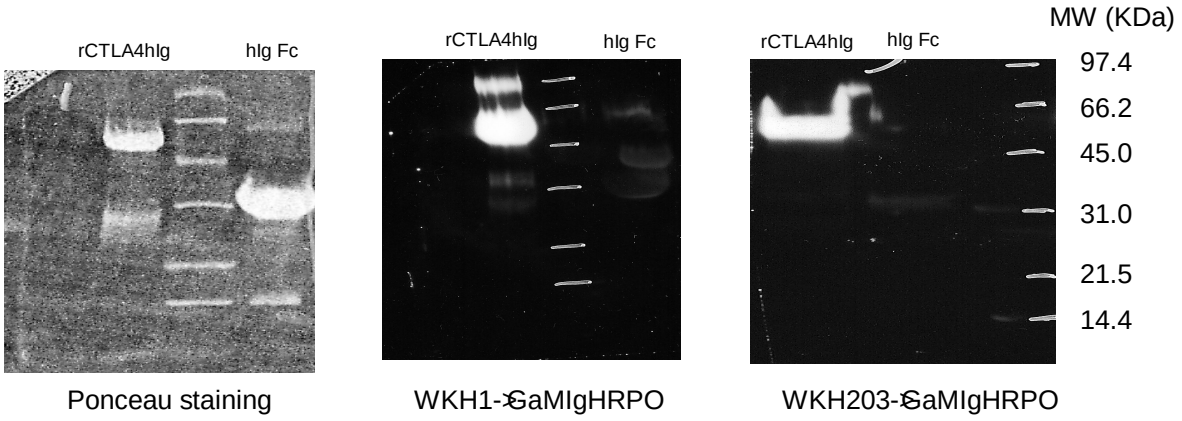
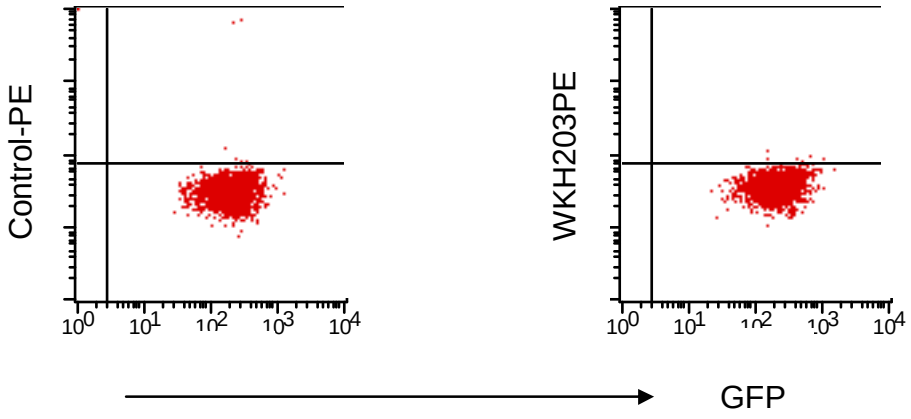


Figure 3. Recognition of rCTLA-4hIg fusion protein by WKH1 and WKH203 in ELISA (A) and western blot (B). 0.5 ug/ml rCTLA-4hIg fusion proteins or commercial human immunoglobulin Fc were coated on ELISA plates, or run on a SDS-PAGE and then transferred to nitrocellulose membrane. Monoclonal antibody WKH1 or WKH203 (both mouse IgG1) was added into the plate or was incubated with the membrane at RT for 60 min to allow them to bind to proteins. Detection of bound antibodies was performed by incubation with enzyme-coupled secondary Abs recognizing mouse IgG1.

BW-GFP cells



BW-rCTLA4-GFP cells

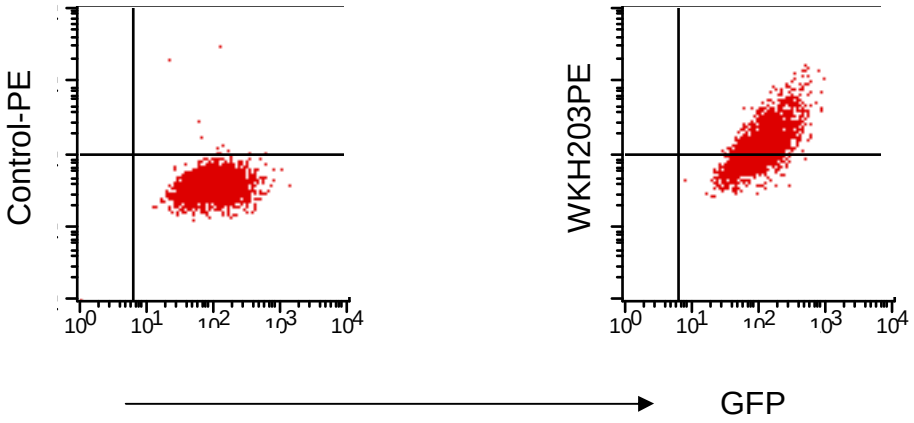


Figure 4. Recognition of rat CTLA-4 by WKH203. BW-cells transduced to express GFP alone, or additionally to express rat CTLA-4 were fixed, permeabilized with 0.5% saponin, and then stained with PE-conjugated WKH203. As negative control, cells were incubated with PE-conjugated control Abs (mouse IgG1).

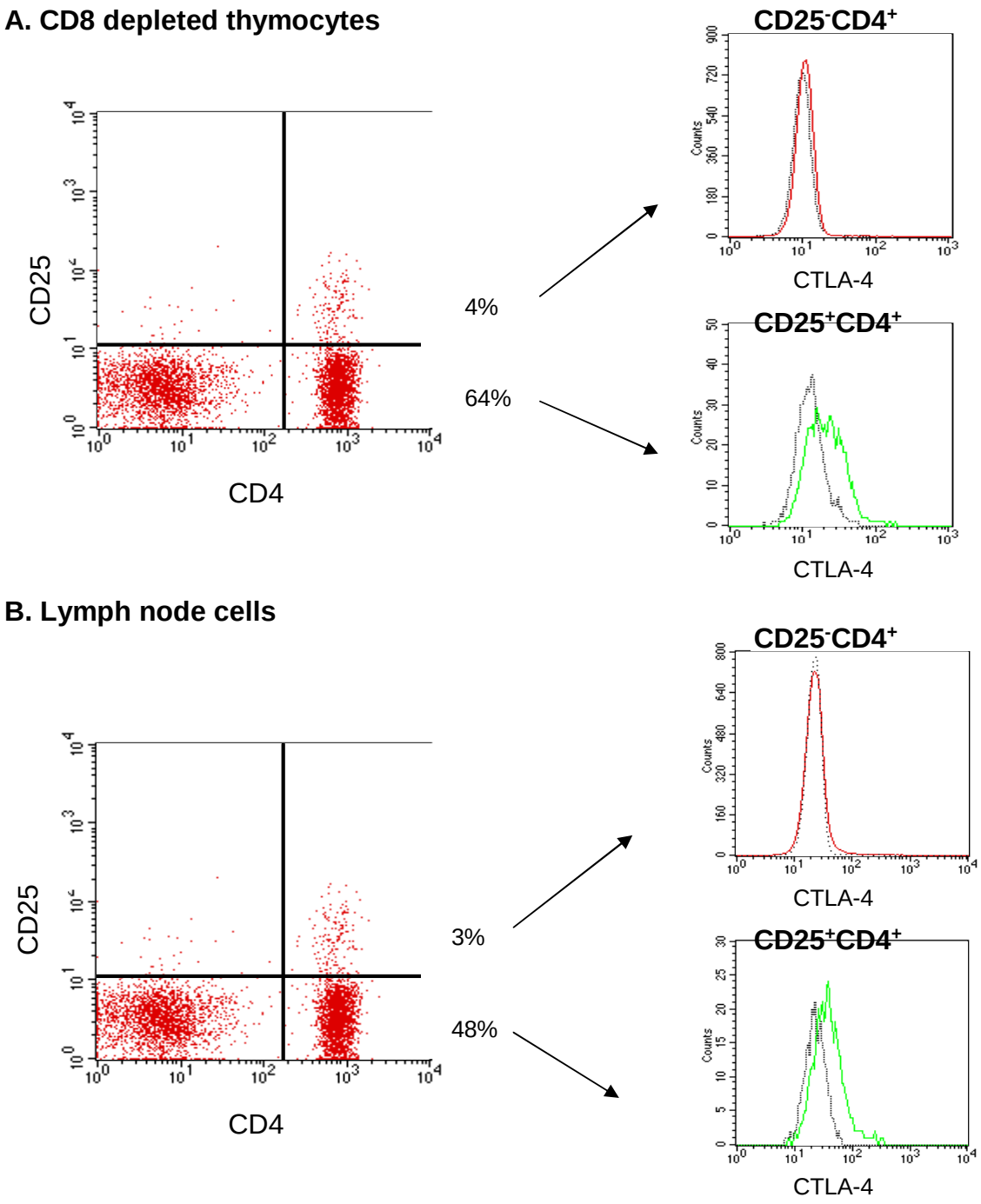


Figure 5. Constitutive expression of CTLA-4 in rat CD25+CD4+ cells from thymus (A) and lymph node (B). $CD8^+$ depleted thymocytes or total lymph node cells were surface stained with CD4 and CD25. Following fixation and permeabilization, cells were i.c. stained with PE-coupled WKH203. For negative control, cells were first incubated with unconjugated WKH203 to block the binding of PE-coupled WKH203 on CTLA-4 molecules. The expression level of CTLA-4 was analysed on T-cell subsets defined by CD4 and CD25.

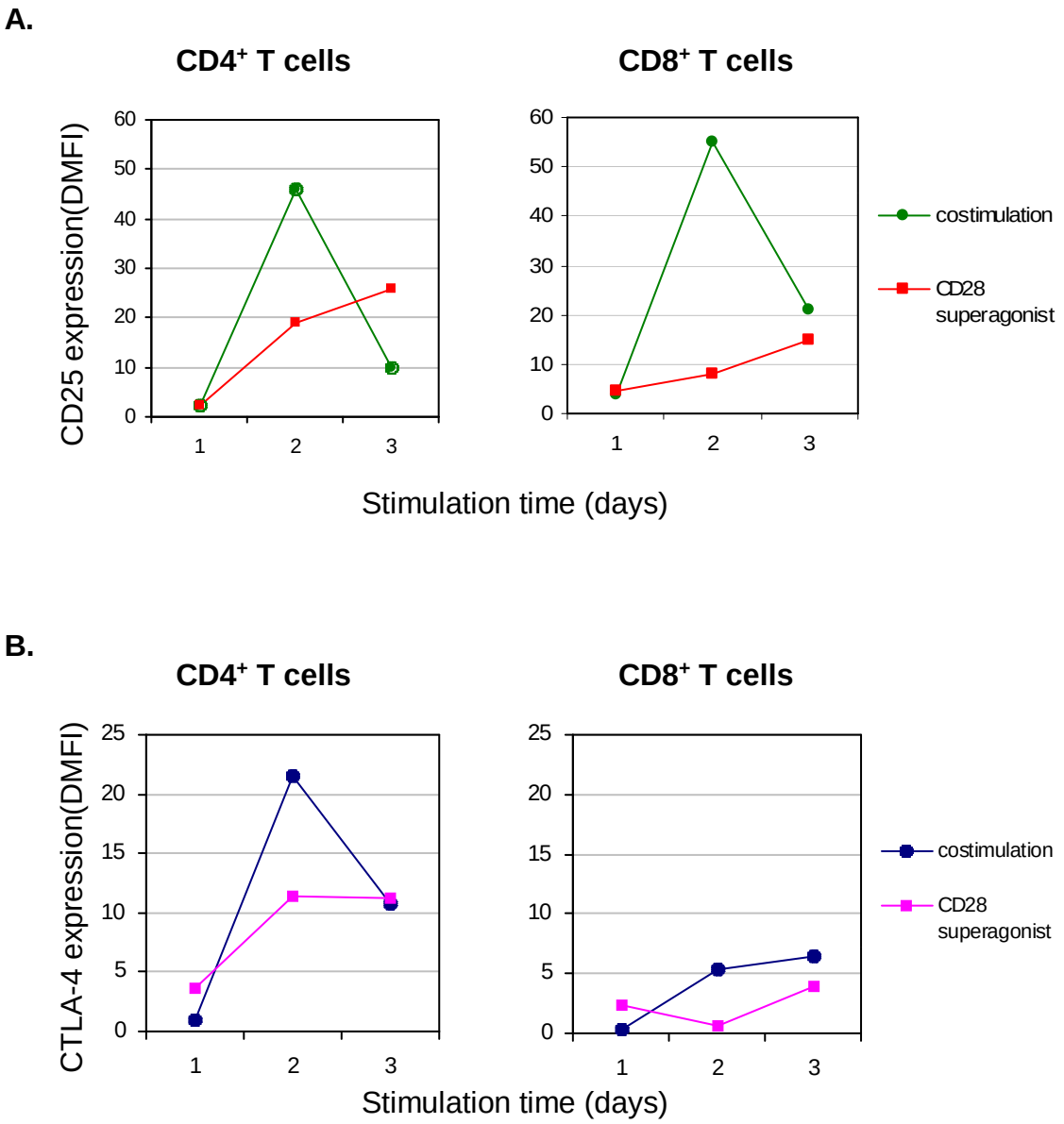


Figure 6. CD25 (A) and CTLA-4 (B) expression by $CD4^+$ versus $CD8^+$ T-cells upon polyclonal stimulation. T lymphocytes were stimulated as indicated, and stained with anti-CD4 and anti-CD25. Following surface staining, cells were stained intracellularlly with PE-coupled anti-rat CTLA-4. CD25 and CTLA-4 expression in $CD4^+$ versus $CD4^-$ ($CD8^+$) T cells were then analysed.

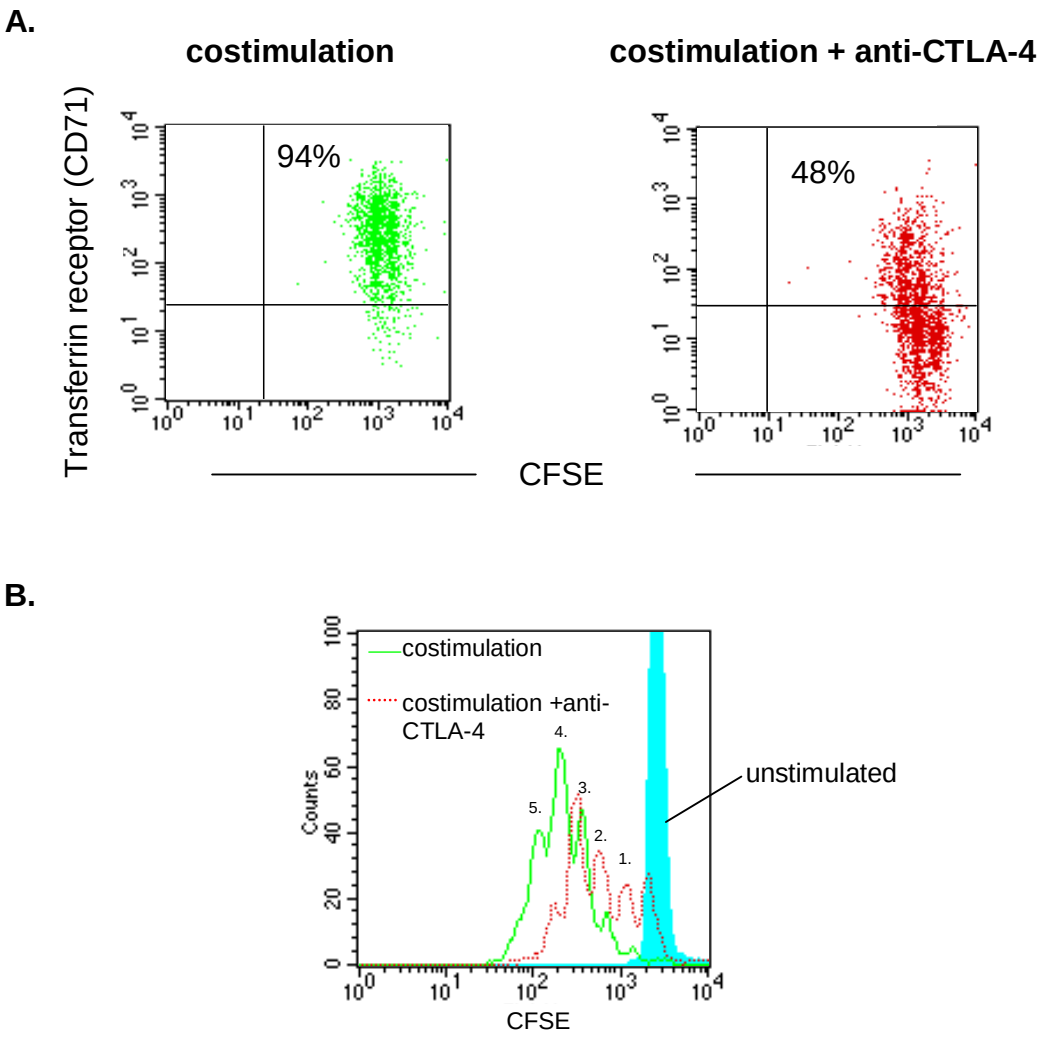


Figure 7. Inhibition of costimulation-induced upregulation of activation marker CD71 (transferrin receptor, A) and cell divisions (B) by cross-linking CTLA-4. CFSE-labelled T-lymphocytes (1x106/ml) were stimulated with the same number of latex beads coated with anti-TCR, anti-CD28, and either a control mouse IgG1 (co-stimulation) or anti-rat CTLA-4 (costimulation +CTLA-4). The ratio of anti-TCR: anti-CD28: control mouse IgG1 or anti-rat CTLA-4 was 1:1:9. Cells were harvested after 48 hour stimulation, stained with anti-CD71, and analysed for CD71 expression and the numbers of cell divisions.

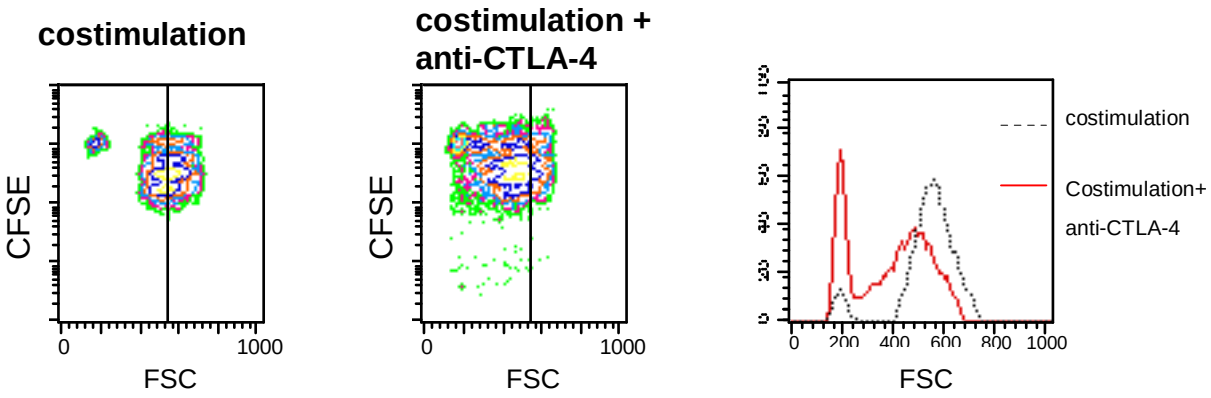


Figure 8. Reduction in cell size of costimulated T cells by cross-linking CTLA-4. CFSE-labelled T cells were cultured with latex beads (cells: beads=1:1) coated with anti-TCR, anti-CD28, and in the presence of control Abs or anti-CTLA-4 as described in Fig. 7. After 48 hour stimulation, cells were harvested and their forward scatter was analysed by flow cytometry.

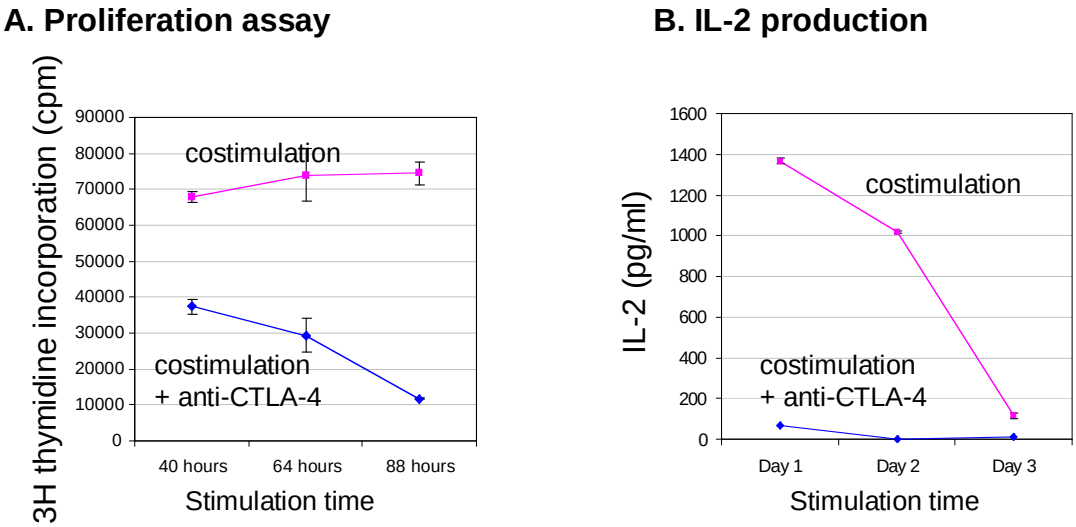
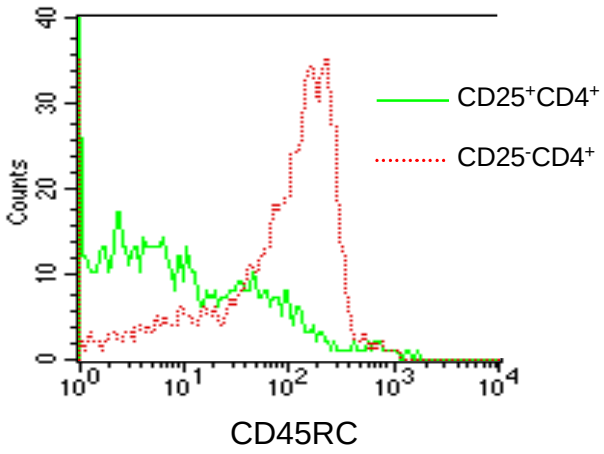


Figure 9. Reduced cell proliferation and IL-2 production in the presence of anti-CTLA-4 mAbs. Pooled T cells were plated in 96 well plates and stimulated with mAbs immobilized on latex beads as described in Fig. 8. (A) For proliferation assays, cells were pulsed with 3H-thymidine for the last 16 hours and harvested at the times indicated. (B) Reduction in IL-2 production upon cross-linking CTLA-4. Supernatants from stimulated cells were collected at indicated times, and IL-2 concentration in supernatants was determined by IL-2 ELISA.

A.



B.

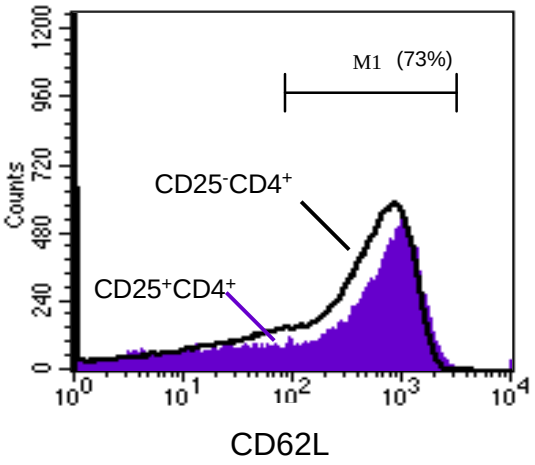
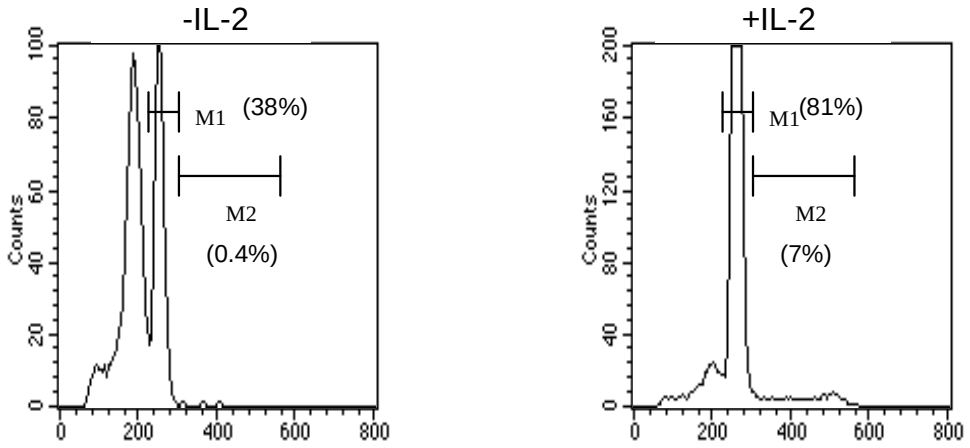


Figure 10. Phenotype of rat CD25$^+$CD4$^+$ versus CD25$^-$CD4$^+$ cells. Pooled lymph node cells from untreated LEW rats were surface stained with anti-CD4, anti-CD25, and examined of their expression of CD45RC (A) and L-selectin (CD62L, B).

A. CD25^{+}CD4^{+} cells



B. CD25^{-}CD4^{+} cells

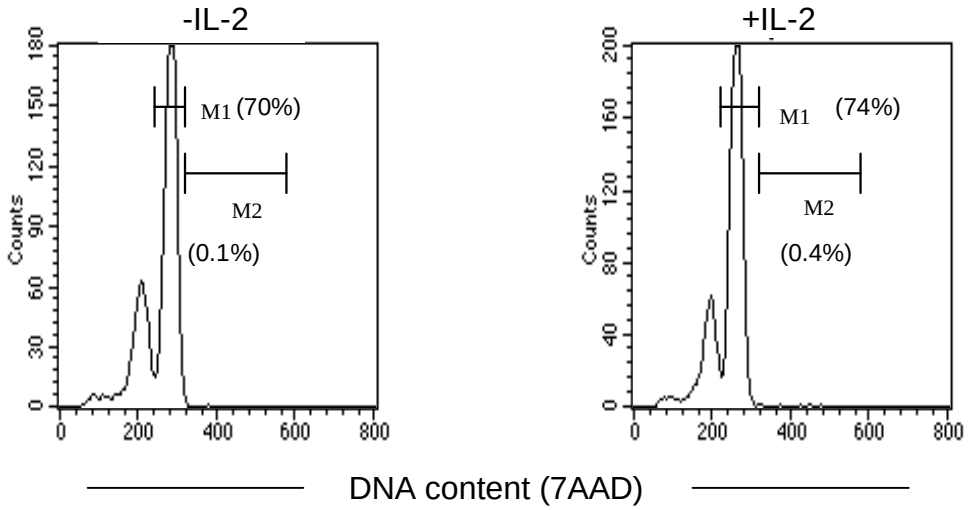


Figure 11. CD25^{+}CD4^{+} cells are prone to cell death *in vitro* and are rescued by exogenous IL-2. CD25^{+}CD4^{+} (A) and CD25^{-}CD4^{+} cells (C) were purified from 6-8 wk healthy rats and cultured in medium in the absence or in the presence of exogenous IL-2 for 2 days. Cells were harvested and incubated with 7AAD in 0.1% saponin for 30 min at RT. DNA content of individual cells was analysed by flow cytometry directly without further washing.

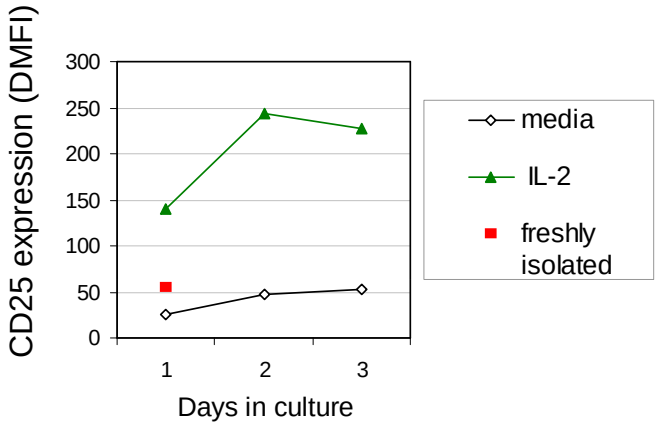


Figure 12. **Increased CD25 expression on $CD25^+CD4^+$ cells cultured with exogenous IL-2.** Purified $CD25^+CD4^+$ T cells were cultured in medium with or without exogenous IL-2. At indicated times, cells were harvested and surface stained with anti-CD25 to determine the CD25 expression level.

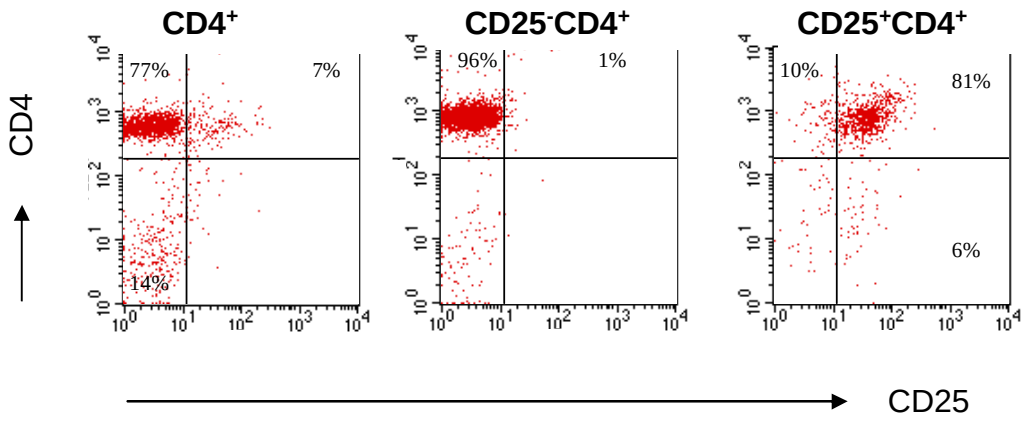


Figure 13. $CD4^+$, $CD25^-CD4^+$ and $CD25^+CD4^+$ cells after MACS purification. Pooled rat lymph node cells were separated in three populations: $CD4^+$ cells, $CD25^-CD4^+$, and $CD25^+CD4^+$ cells.

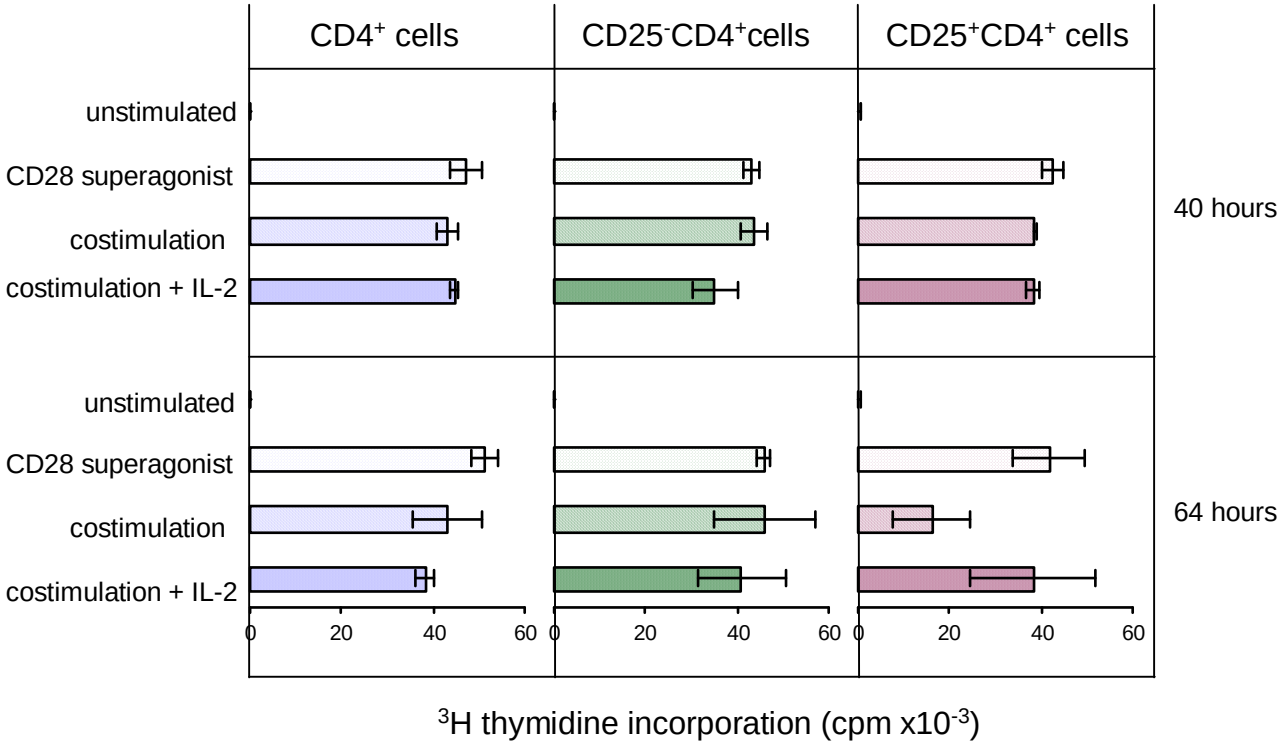


Figure 14. Sustained CD25$^+$CD4$^+$ cell proliferation upon CD28 superagonist stimulation. Purified CD4$^+$, CD25$^-$CD4$^+$, and CD25$^+$CD4$^+$ T cells were stimulated either with plate-bound anti-TCR plus anti-CD28 or with CD28 superagonist in the absence or in the presence of IL-2. Cells were pulsed with 3H-thymidine for the last 16 hours and harvested at the indicated time.

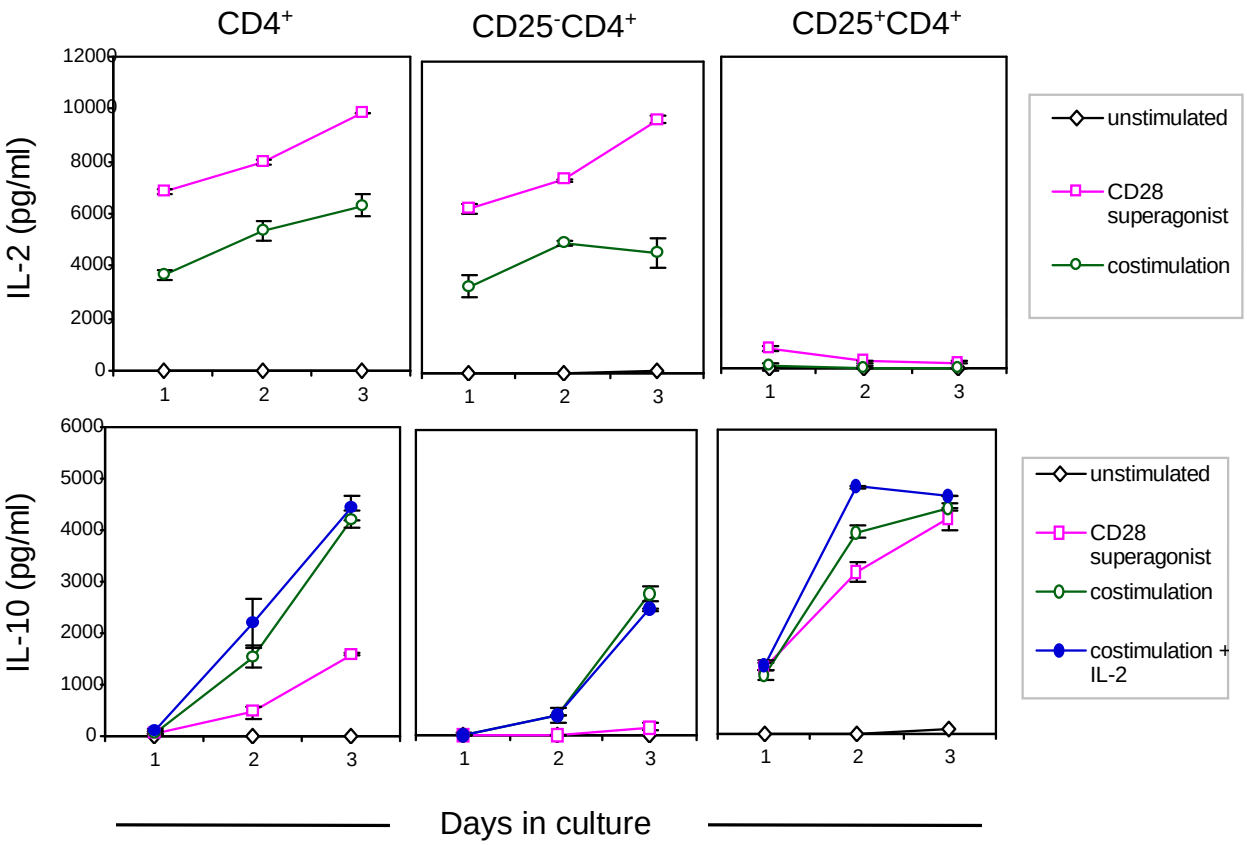


Figure 15. IL-2 and IL-10 production of naive CD25$^-$CD4$^+$ versus CD25$^+$CD4$^+$ cells upon stimulation. Isolated CD25$^-$CD4$^+$ and CD25$^+$CD4$^+$ cells were stimulated in 96-well plates as described in Fig. 15. At indicated time points, supernatants were collected, and the concentrations of IL-2 and IL-10 were determined by ELISA. Results are presented as average concentration of triplicates +/- SD.

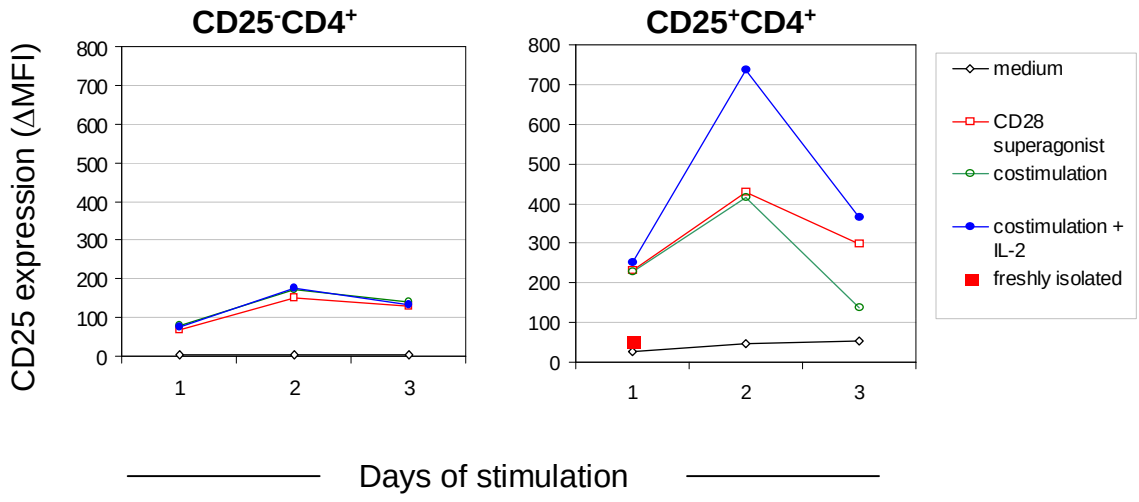


Figure 16. Kinetics of CD25 expression on $CD25^{-}CD4^{+}$ versus $CD25^{+}CD4^{+}$ cells upon stimulation. Purified naive $CD25^{-}CD4^{+}$ (A) and $CD25^{+}CD4^{+}$ T cells (B) were stimulated as described in Fig. 15. At the indicated times, cells were harvested, incubated with NMIg to reduce the unspecific binding of Abs, and then surface stained with anti-CD25. The level of CD25 expression is presented as DMFI, calculated by fluorescence intensity of CD25 staining minus control staining.

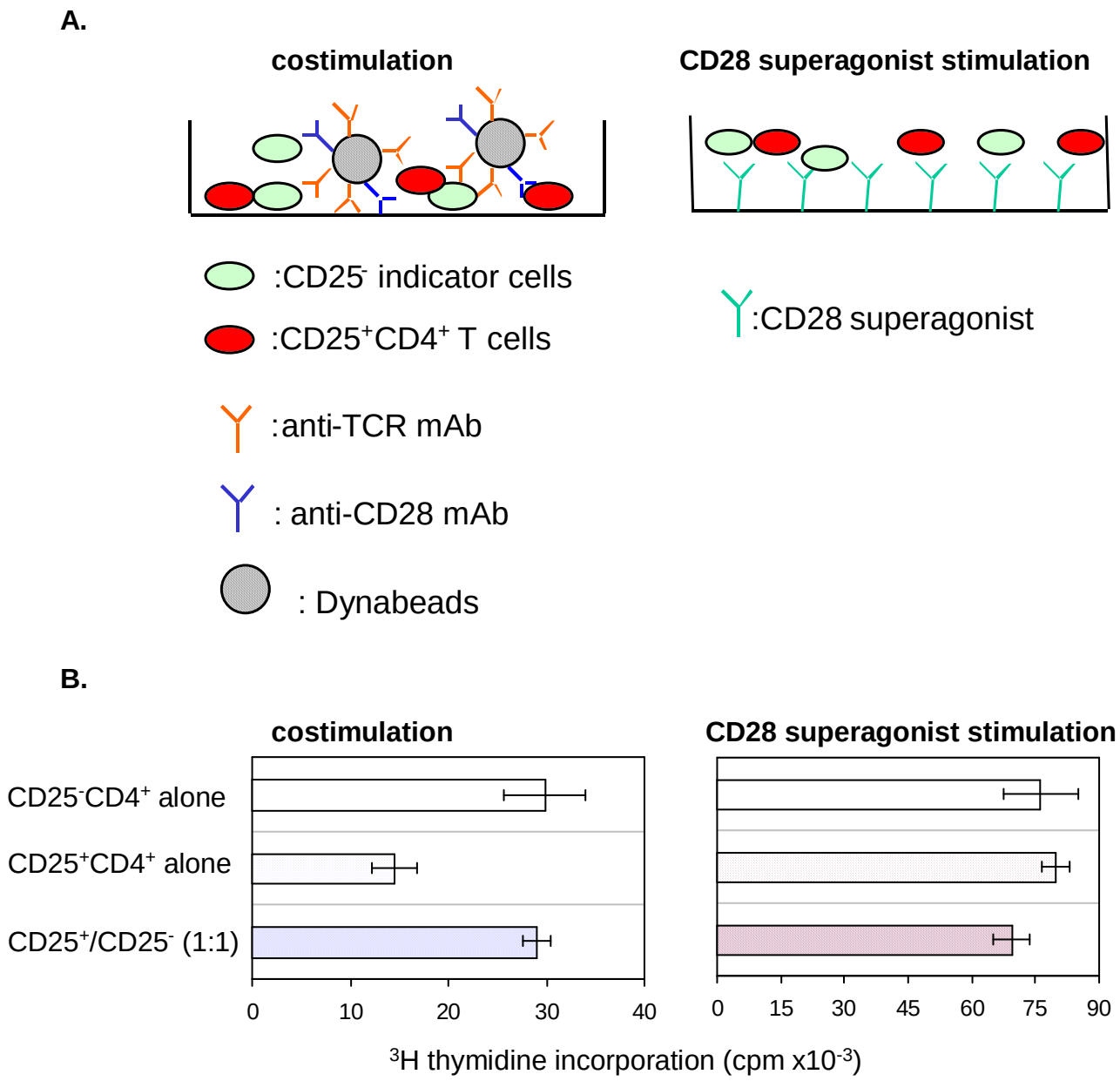
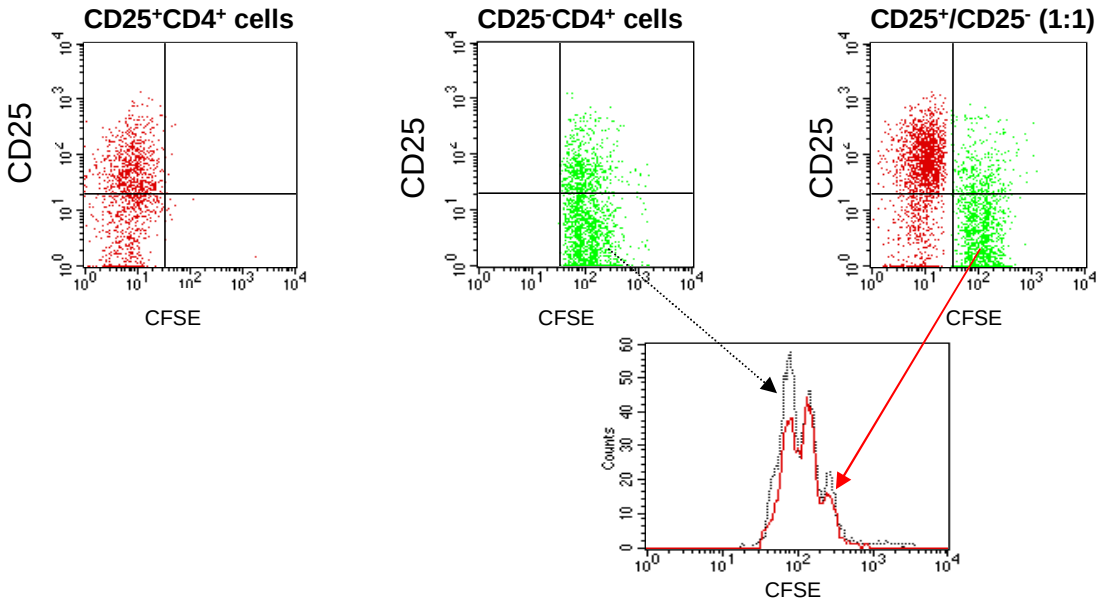


Figure 17. No suppression of CD25$^-$CD4$^+$ cell proliferation in coculture with naive CD25$^+$CD4$^+$ cells. (A.) Experimental settings for examining suppressor activity of CD25+CD4+ cells from untreated rats. (B.) Purified CD25$^-$CD4$^+$ and CD25$^+$CD4$^+$ cells from healthy rats were either cultured alone or, in case of coculture, cultured together with the same number of CD25$^+$CD4$^+$ cells. Cells were stimulated either with anti-TCR plus anti-CD28 or with CD28 superagonist. Cells were pulsed with 3H-thymidine for the last 16 hours and harvested after 64 hour stimulation.

A. Costimulation



B. CD28 superagonist stimulation

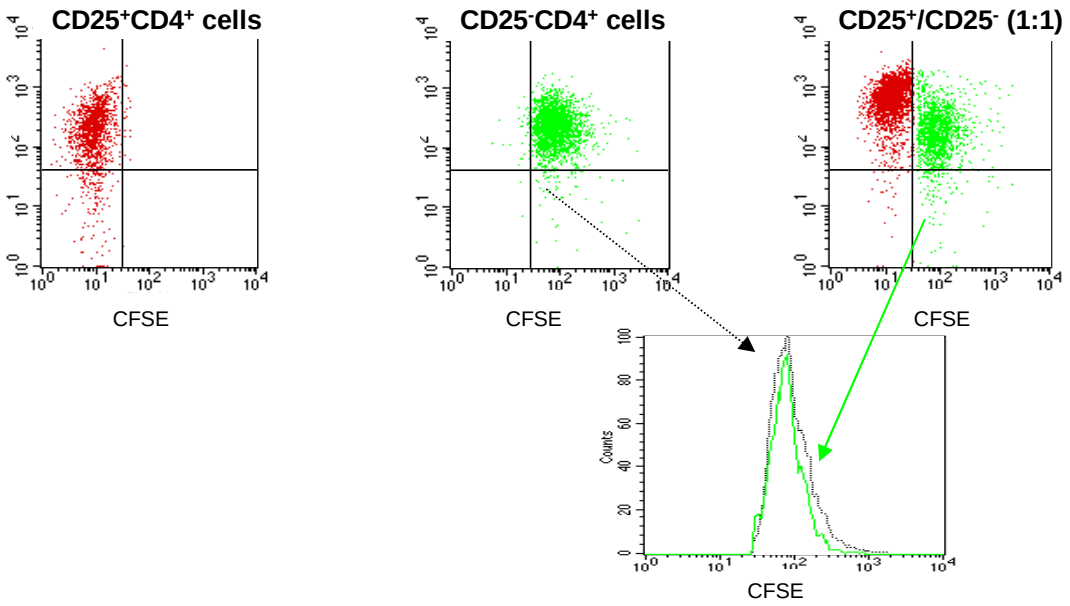


Figure 18. Reduction in cell divisions of costimulated naive indicator CD25$^-$CD4$^+$ cells in coculture with CD25$^+$CD4$^+$ cells. CFSE-labelled naive CD25$^-$CD4$^+$ indicator cells were either cultured alone or in coculture with the same number of CD25$^+$CD4$^+$ cells and stimulated with either anti-TCR plus anti-CD28 (A) or with CD28 superagonist (B). After 2 days, cells were harvested, surface stained with anti-CD25 and analysed for cell divisions and CD25 expression. CFSE-labelled CD25$^-$CD4$^+$ cells were distinguished from CD25$^+$CD4$^+$ cells by their higher fluorescence intensity in FL-1.

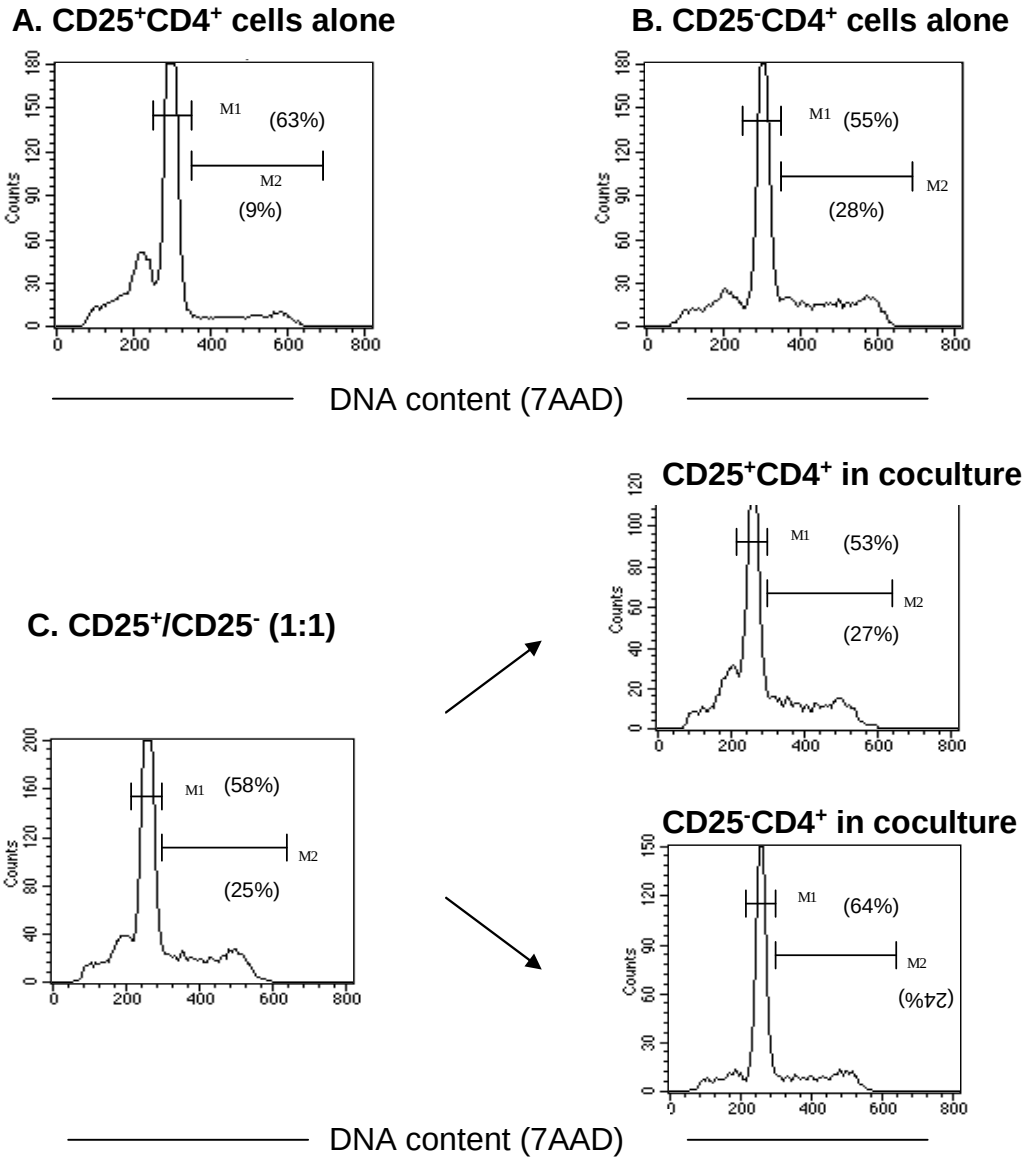


Figure 19. Increased cycling of $CD25^+CD4^+$ cells in coculture with $CD25^-CD4^+$ cells. $CD25^+CD4^+$ and CFSE-labelled $CD25^-CD4^+$ cells were either cultured alone or in coculture with the same number of $CD25^+CD4^+$ cells. After 48 hour costimulation, cells were harvested, permeabilised, and incubated with 7AAD for 30 min. The DNA content of individual cells was then analysed. CFSE labelled $CD25^-CD4^+$ cells were distinguished from $CD25^+CD4^+$ in coculture by their high fluorescence one intensity.

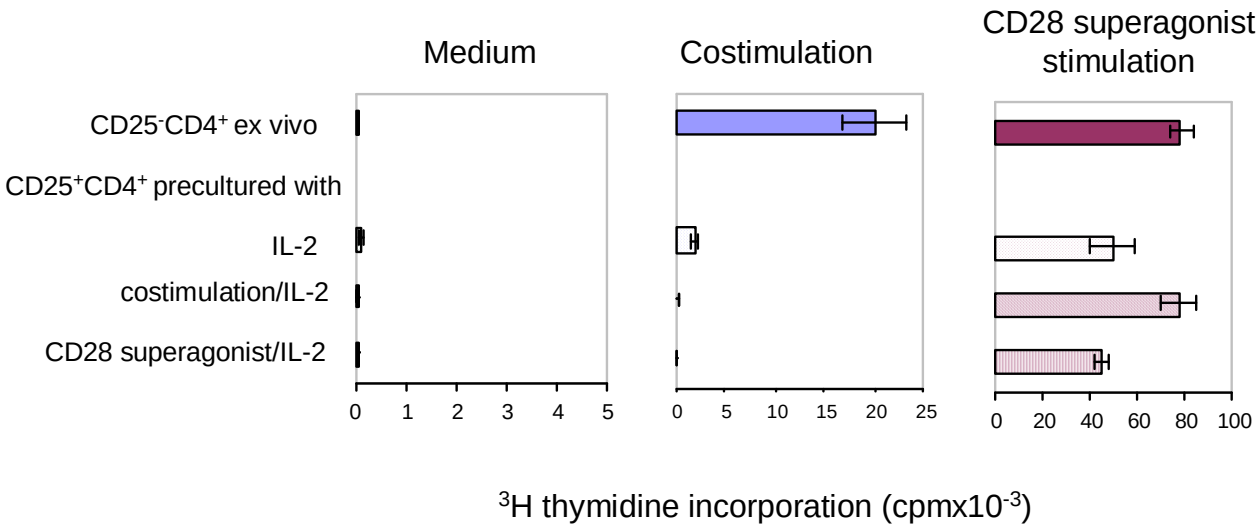


Figure 20. Preactivated CD25$^+$CD4$^+$ cells proliferate poorly to costimulation but respond to CD28 superagonistic restimulation. Freshly isolated CD25$^+$CD4$^+$ cells from untreated rats were either precultured with IL-2 alone (300 U/ml), or additionally with anti-TCR plus anti-CD28 or with CD28 superagonist. After 72 hours, cells were harvested, washed twice, and stimulated either with plate-bound anti-TCR plus anti-CD28 (costimulation) or with CD28 superagonist for 3 days. Cells were pulsed with 3H-thymidine for the last 16 hours.

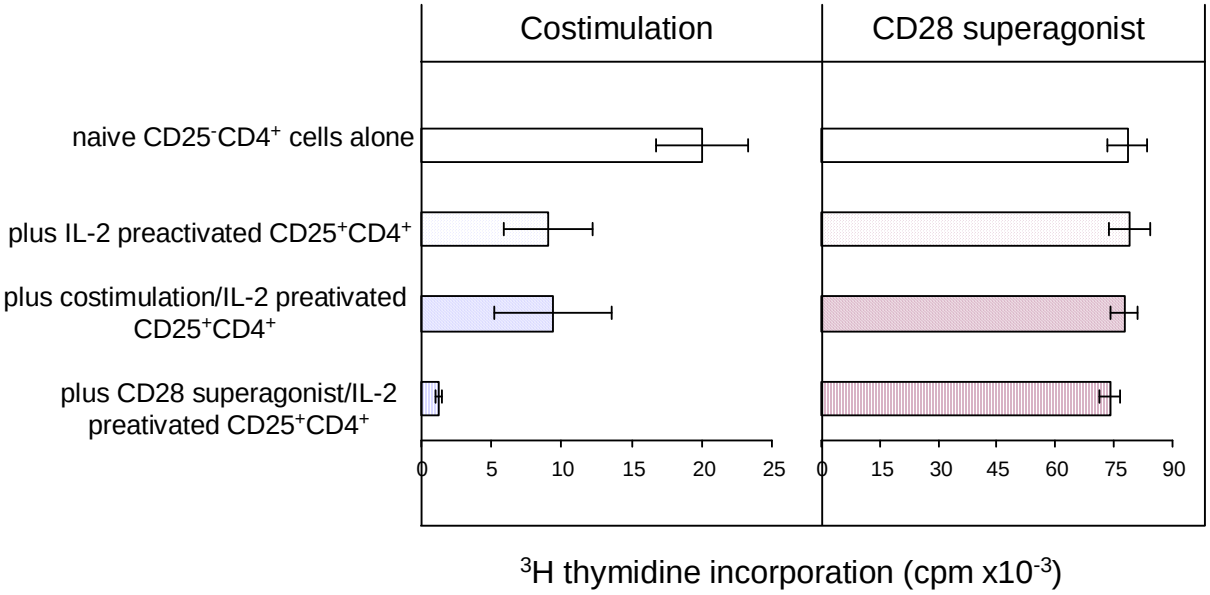


Figure 21. Reduced proliferation in coculture of indicator cells with pre-activated $CD25^+CD4^+$ cells upon costimulation but not upon CD28 superagonist stimulation. Purified naive $CD25^-CD4^+$ T cells were either cultured alone, or in coculture with pre-activated $CD25^+CD4^+$ cells (as described in Fig. 20) at the ratio of 1:1. All wells contained the same number of cells. Cells were then stimulated with anti-TCR plus anti-CD28 (ratio 2:1) coated on Dynabeads. After 48 hours, cells were pulsed with 3H- thymidine and harvested for further 16 hours.

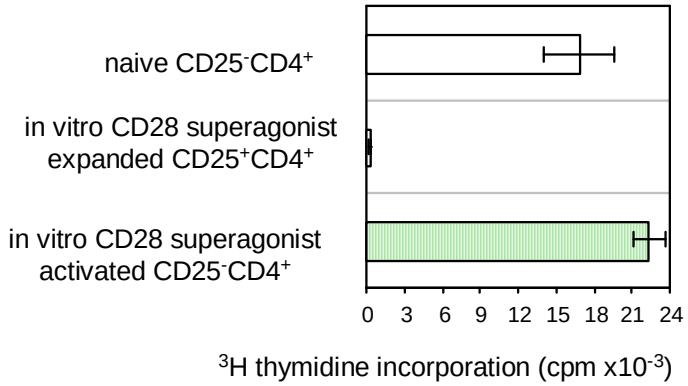


Figure 22. CD28 superagonist preactivated CD25$^-$CD4$^+$ cells but not CD25$^+$CD4$^+$ cells are activated to proliferate by following costimulation. Freshly isolated CD25$^-$CD4$^+$ cells or CD25$^+$CD4$^+$ cells were first activated with CD28 superagonist and IL-2 (300 U/ml) for 3 days. Harvested cells were then re-stimulated (5x104/well) with plate-bound anti-TCR plus anti-CD28 (costimulation). After 48 hours of culture, cells were pulsed with 3H-thymidine and harvested after further 16 hours stimulation.

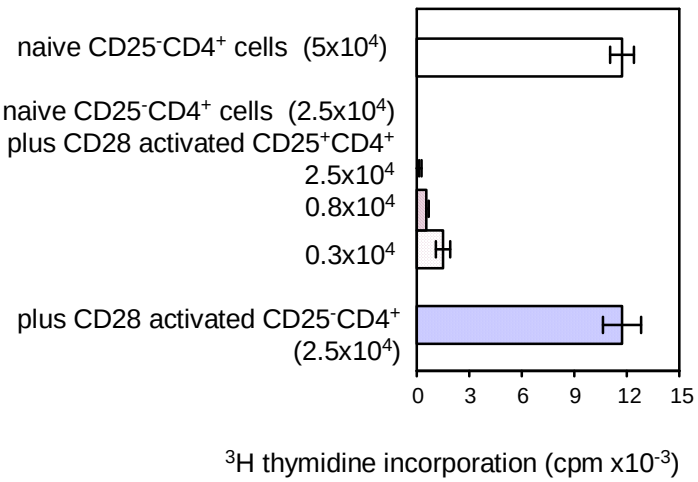


Figure 23. Inhibition of costimulation induced proliferation by preactivated CD25$^+$CD4$^+$ cells, but not by preactivated CD25$^-$CD4$^+$ cells. Freshly isolated CD25$^-$CD4$^+$ cells or CD25$^+$CD4$^+$ cells were pre-activated with CD28 superagonist plus IL-2 for 3 days. For examining their suppressor activity, preactivated cells were cocultured with the same number of naive CD25$^-$CD4$^+$ cells and stimulated with anti-TCR plus anti-CD28. CD25$^-$CD4$^+$ cells cultured alone served as positive control. Cells were pulsed for the last 16 hours with 3H-thymidine, and harvested after 64 hour stimulation.

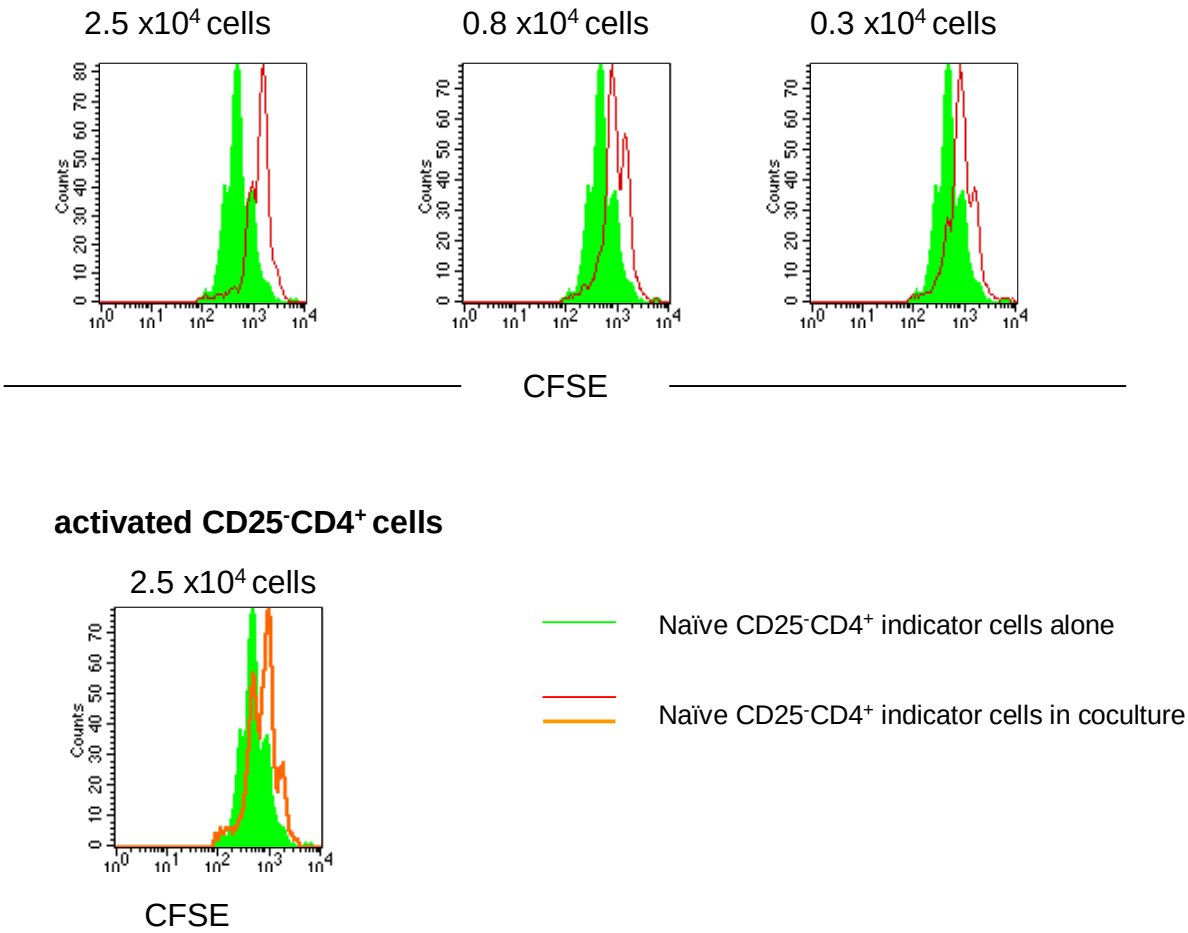


Figure 24. Reduction in divisions of CD25$^-$CD4$^+$ cells in coculture with activated CD25$^-$ or CD25$^+$CD4$^+$ T cells. CFSE-labelled naive CD25$^-$CD4$^+$ indicator cells were either cultured alone, or with preactivated CD25$^-$CD4$^+$ cells or CD25$^+$CD4$^+$ cells as indicated. The cell divisions undergone by CD25$^-$CD4$^+$ cells were examined by CFSE dilution.

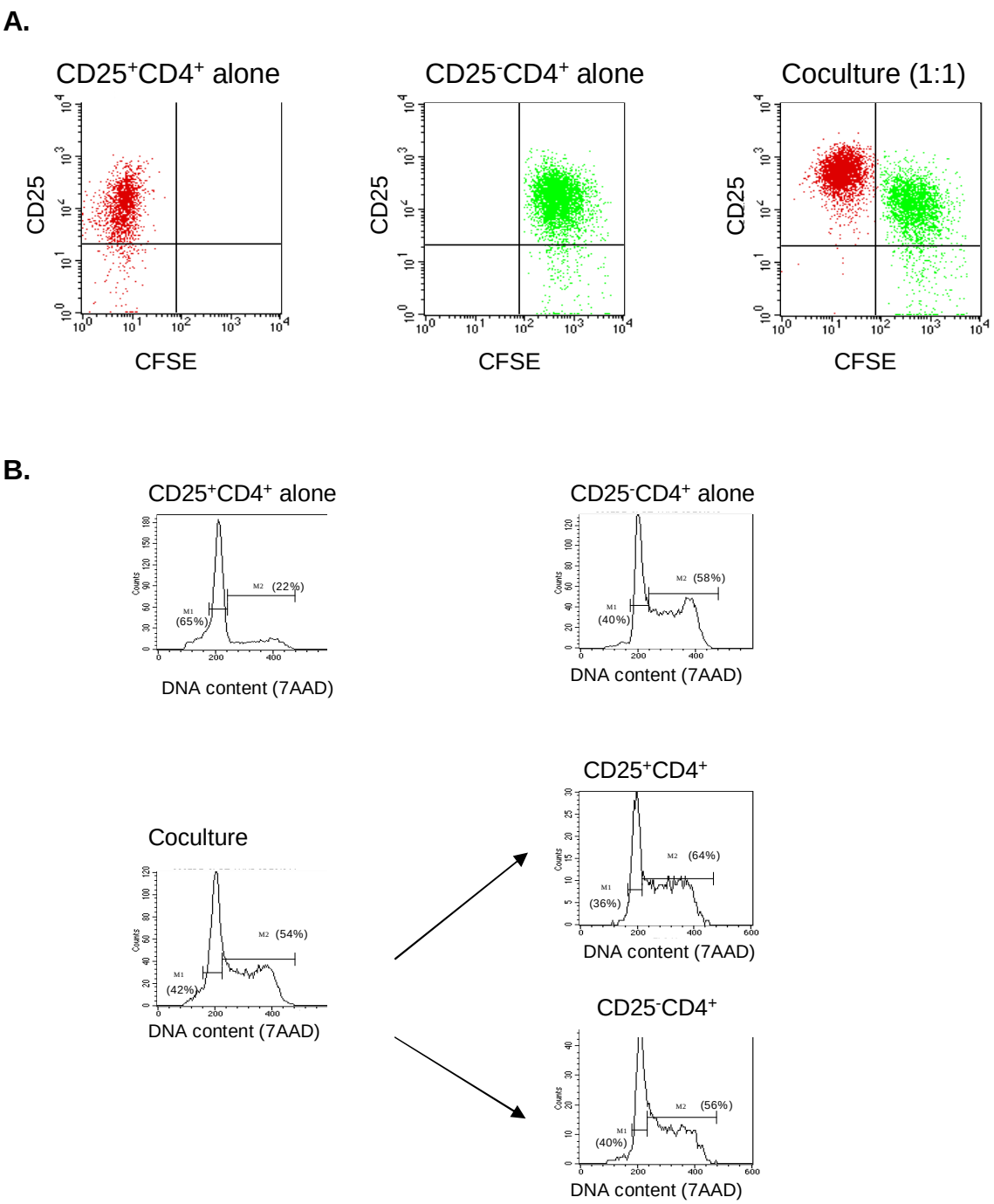


Figure 25. No suppression in CD25 expression, cell divisions, and cell cycling of CD25$^-$CD4$^+$ indicator cells cocultured with preactivated CD25$^+$CD4$^+$ cells upon CD28 superagonist stimulation. CFSE-labelled naive CD25$^-$CD4$^+$ cells were either cultured alone, or in coculture with the same number of CD28-superagonist preactivated CD25$^+$CD4$^+$ cells. Cells were then stimulated with CD28 superagonist for 48 hours, and analysed for their CD25 expression and DNA content.

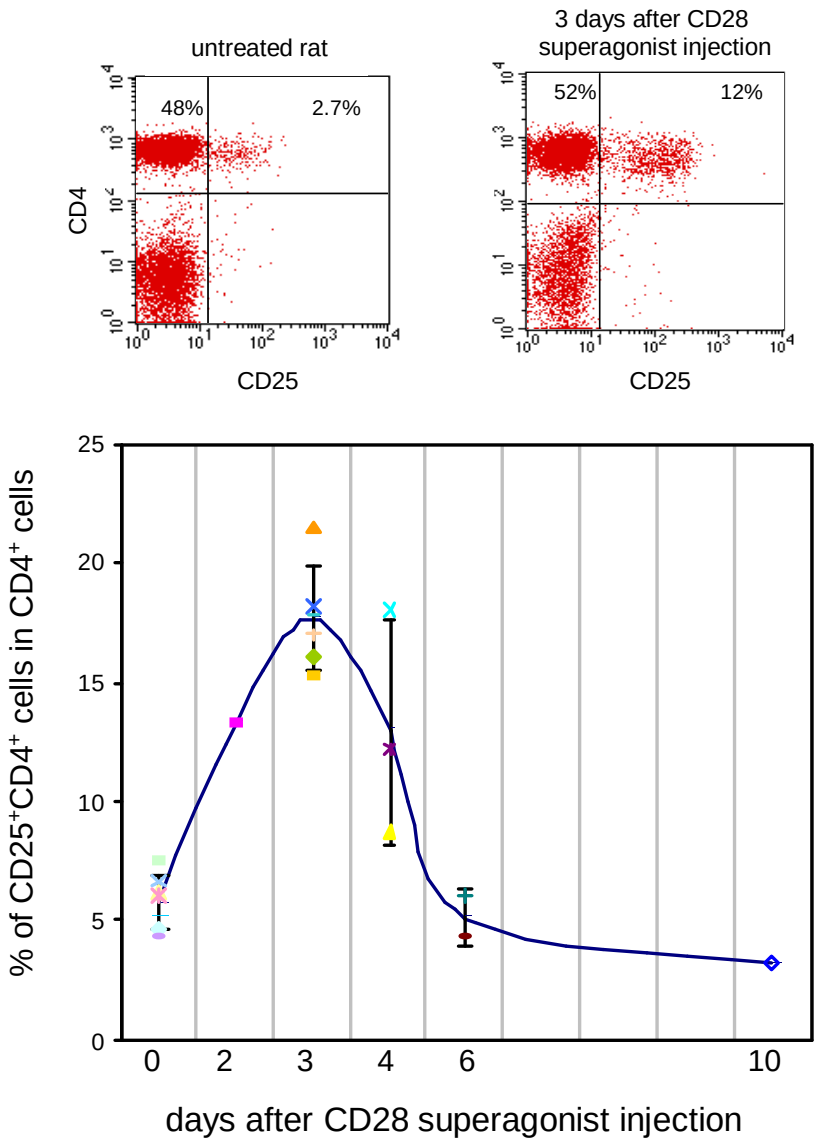


Figure 26. Transient increase of $CD25^+CD4^+$ cell percentage upon CD28 superagonist injection in rats. (A). Percentage of $CD25^+CD4^+$ cells in lymph node cells from untreated rats versus LN cells from rats 3 days after CD28 superagonist injection. (B). Kinetics of $CD25^+CD4^+$ cell percentage in $CD4^+$ cells upon CD28 superagonist injection. Pooled lymph node cells were taken from rats at indicated times after CD28 superagonist injection, and surface stained with anti-CD4 and anti-CD25 mAbs.

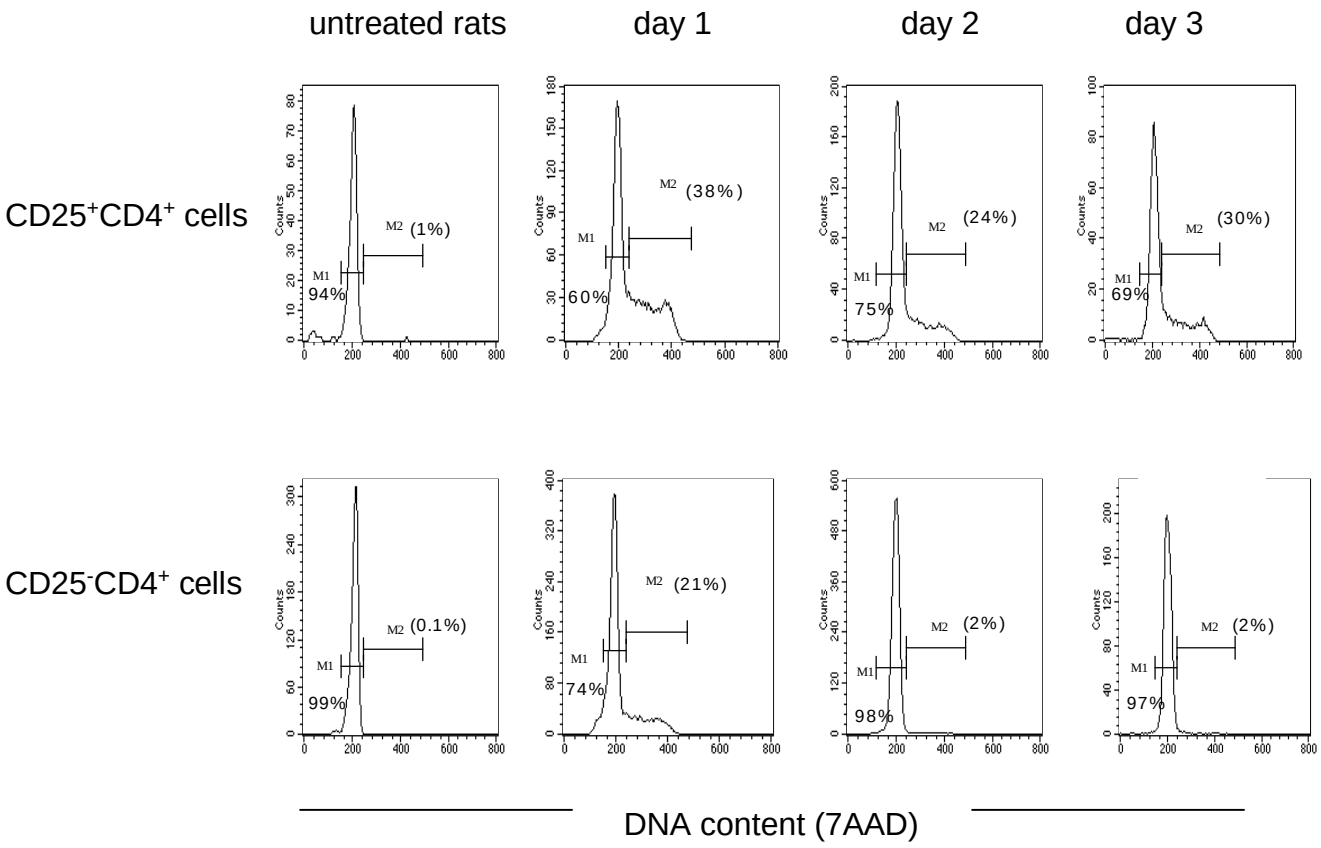


Figure 27. Percentage of proliferating cells of CD25$^-$CD4$^+$ versus CD25$^-$CD4$^+$ cells upon CD28 superagonist injection. Pooled lymph node cells collected from CD28 superagonist treated rats at the times indicated were surface stained with anti-CD4 and anti-CD25. After washing, cells were incubated with 7AAD in 0.1% saponin. DNA content of individual cells was gated by surface marker expression.

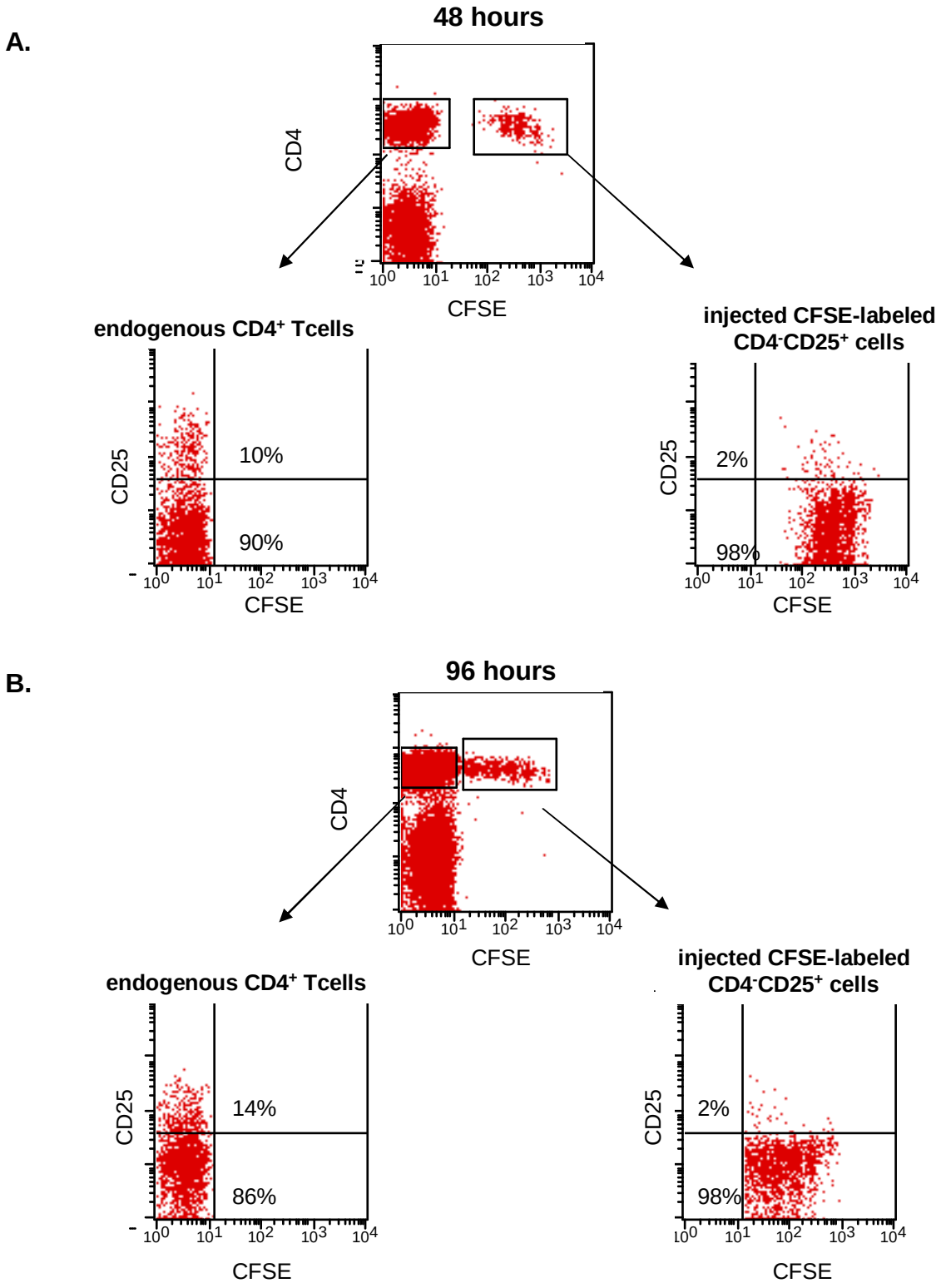
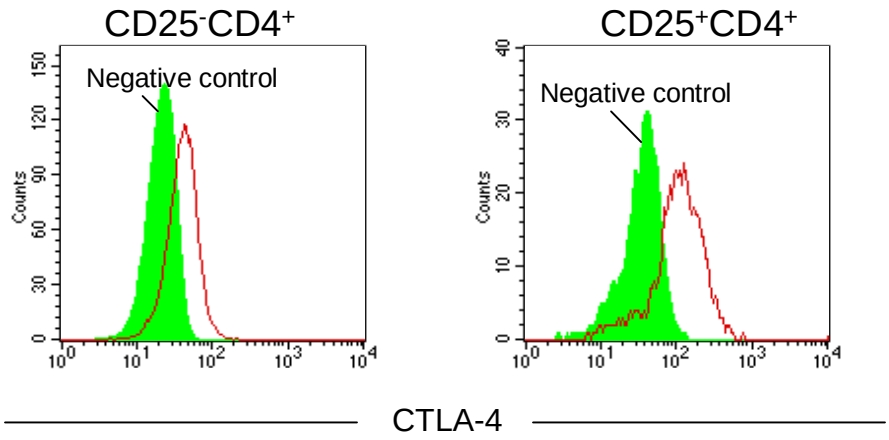


Figure 28. CD25 is not expressed on transferred CD25$^-$CD4$^+$cells upon *in vivo* CD28 superagonist stimulation. 2x107 CD25$^-$CD4$^+$ T cells from peripheral and mesenteric LN were labelled with CFSE and injected i. v. into host rats. Eight hours later, rats received i. v. 1 mg CD28 superagonist. Expression of CD25 in pooled lymph node cells were analysed 48- (A) and 96 hours (B) after injection of CD28 superagonist.

A. CTLA-4 expression



B. CD45RC expression

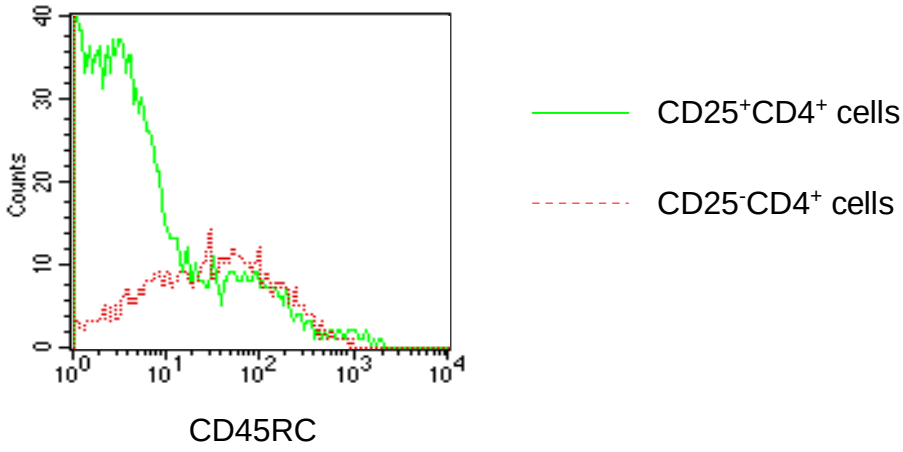


Figure 29. CTLA-4 and CD45RC expression of CD25$^+$CD4$^+$ versus CD25$^-$CD4$^+$ cells from CD28 superagonist injected rats. Pooled lymph node cells were collected from rats 3 days after CD28 superagonist treatment, and surface stained with anti-CD4, anti-CD25, and anti-CD45RC mAbs (B). For detection of CTLA-4 expression (A) in different cell populations, cells were fixed and permeabilised for intracellular staining with PE- conjugated anti-rat CTLA-4 after surface staining with anti-CD4 and anti-CD25.

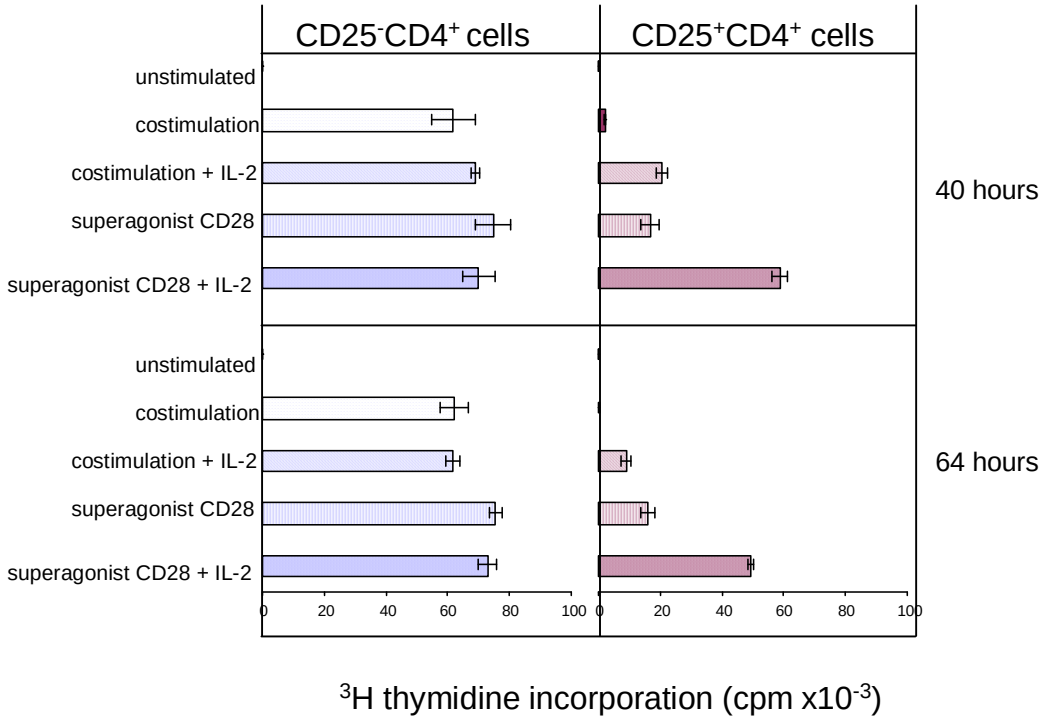


Figure 30. CD25$^+$ but not CD25$^-$CD4$^+$ T cells from CD28 superagonist treated rats proliferate poorly upon *in vitro* costimulation. CD25$^-$CD4$^+$ cells and CD25$^+$CD4$^+$ cells isolated from CD28 superagonist treated rats were subjected to in vitro restimulation with either anti-TCR plus anti-CD28 or with CD28 superagonist in the absence or in the presence of IL-2. Cells were pulsed for the last 16 hours and harvested at indicated time points.

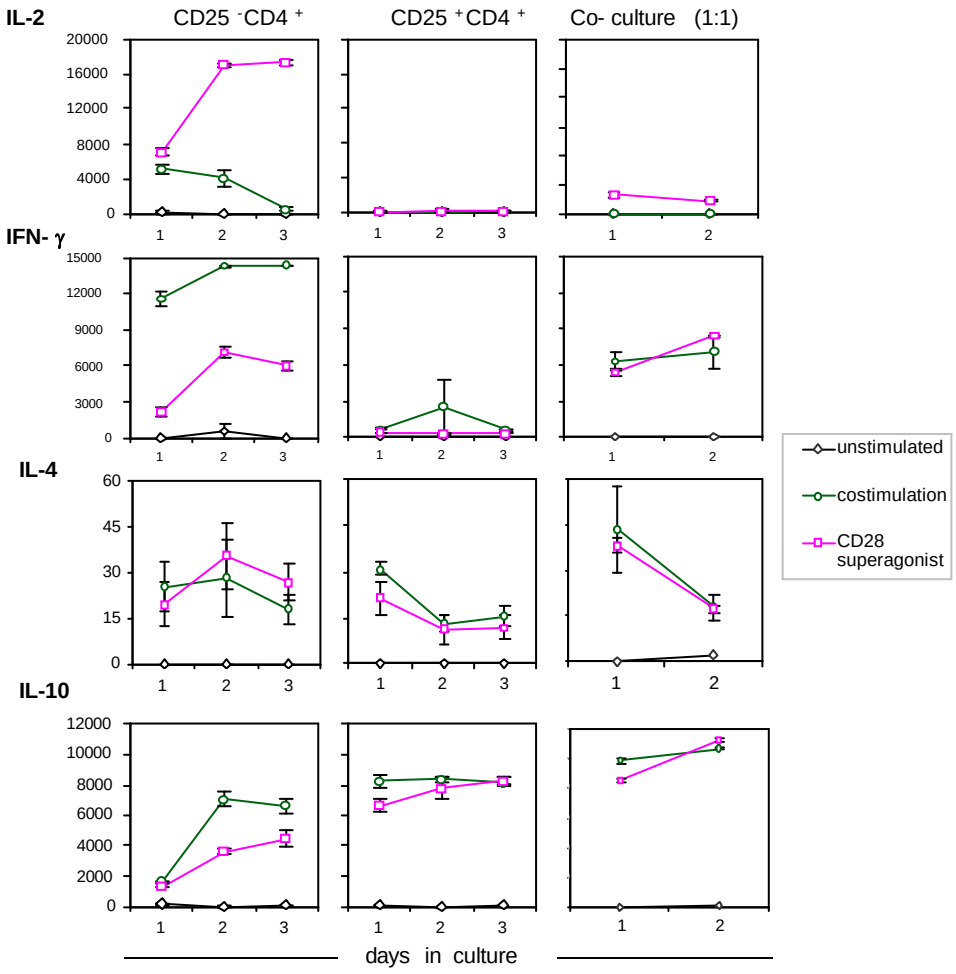


Figure 31. Cytokine profile of cells from CD28 superagonist treated rats. $CD25^{-}CD4^{+}$ cells and $CD25^{+}CD4^{+}$ cell were isolated from rats treated with CD28 superagonist 3 days before, and cultured either alone or cultured together at the ratio of 1:1. Cells were either re-stimulated with plate-bound anti-TCR plus soluble anti-CD28 (co-stimulation), or with CD28 superagonist. At indicated time points, supernatants were collected, and the concentration of IL-2, IFN-g, IL-4 and IL-10 were determined by ELISA.

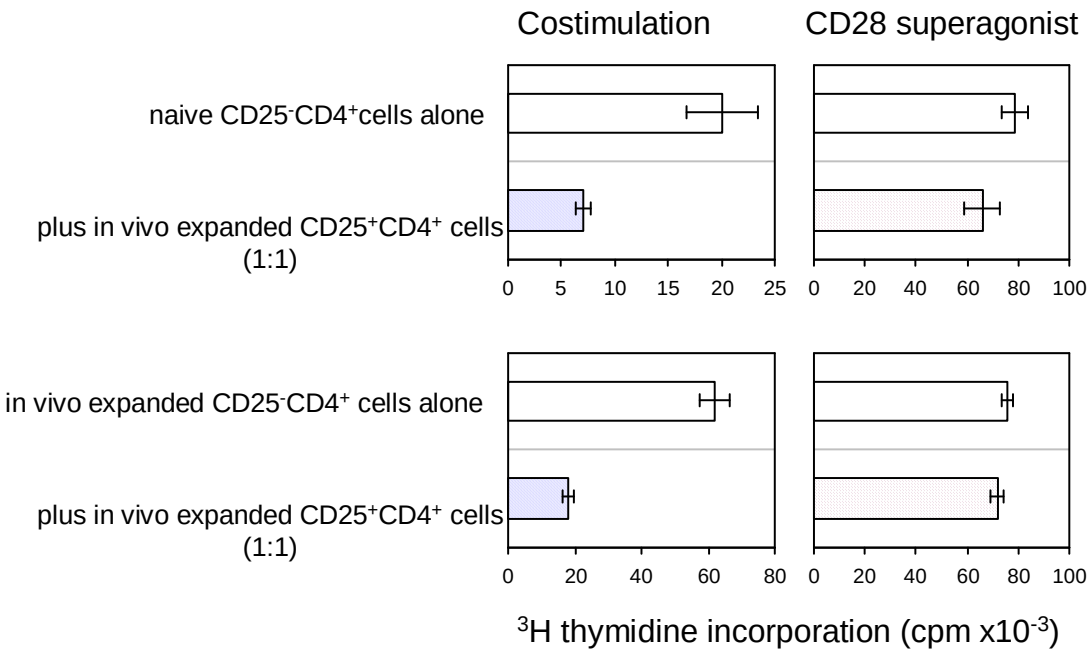


Figure 32. Inhibition of costimulation induced proliferation but no effect on CD28 superagonist induced proliferation by $CD25^+CD4^+$ cells from CD28 superagonist injected rats. $CD25^+CD4^+$ cells from rats after CD28 superagonist treatment were isolated as suppressor cells. Naive $CD25^-CD4^+$ cells (A and B) or $CD25^-CD4^+$ cells from the same rats where the suppressor cells were isolated (C and D) served as indicator cells. The same number of indicator cells and suppressor cells were cultured together. Indicator cells cultured alone were served as control. Cells were either stimulated with anti-TCR plus anti-CD28 (A and C), or with CD28 superagonist (B and D). After 48 hour stimulation, 3H-thymidine was added into each well, and cells were harvested after further 16 hour culture.

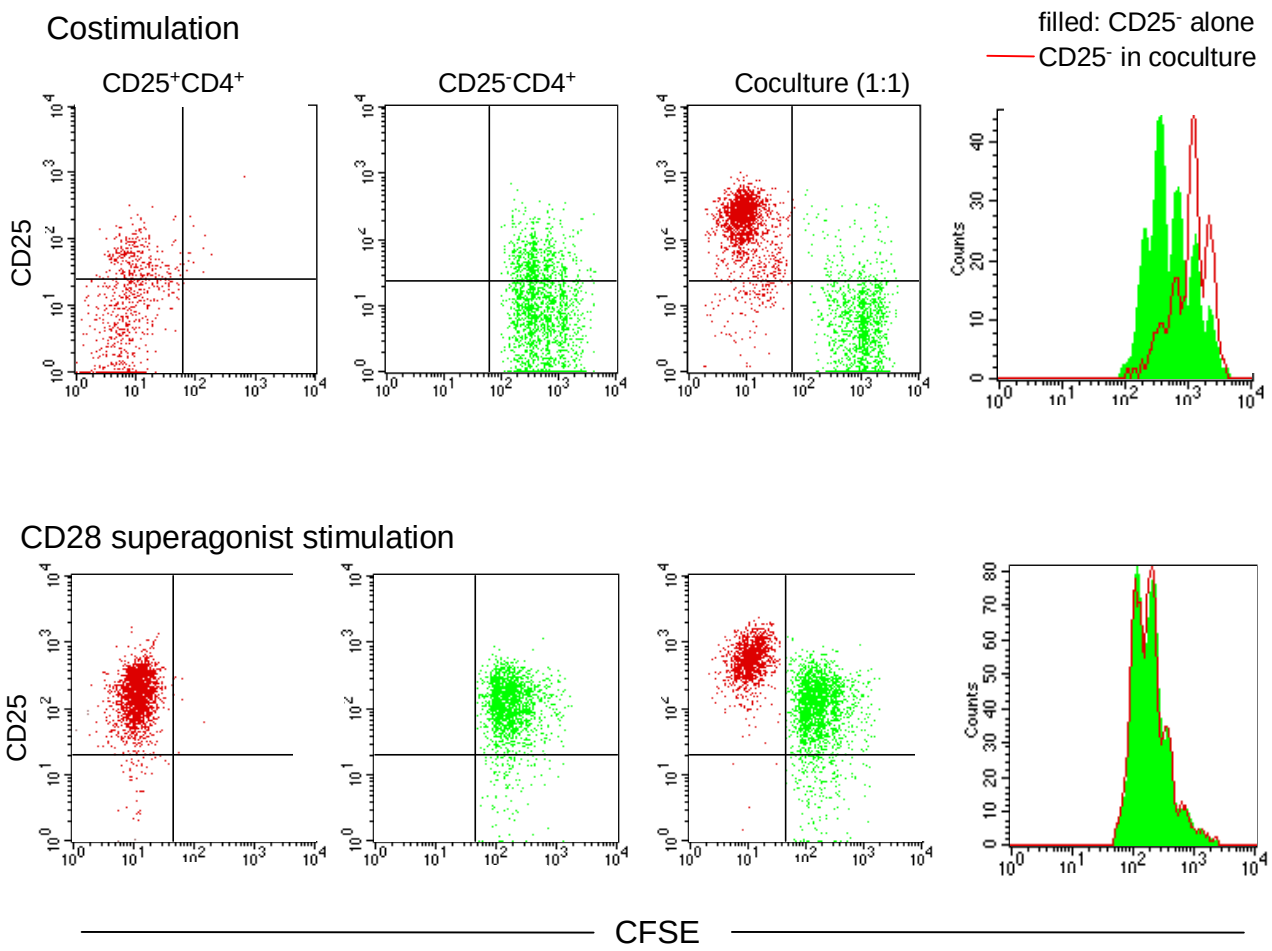


Figure 33. Reduced CD25 expression and cell divisions of $CD25^{-}CD4^{+}$ indicator cells in coculture with *in vivo* activated $CD25^{+}CD4^{+}$ cells upon costimulation (A) but not under CD28-superagonist stimulation (B). CFSE-labelled, naive $CD25^{-}CD4^{+}$ indicator cells were either cultured alone, or cocultured with $CD25^{+}CD4^{+}$ cells from CD28 superagonist treated rats at the ratio of 1:1. Cells were either stimulated with anti-TCR plus anti-CD28 (costimulation, A), or stimulated with CD28 superagonist (B). After 48 hours, CD25 expression of each cell population was examined. Cell divisions undergone by $CD25^{-}CD4^{+}$ indicator cells were examined according to their CFSE fluorescence intensity.