

CD4⁺CD25⁺ Regulatory T Cells Preventing Detrimental Autoimmune Reactions

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Abstract: *Objective:* Autoimmune diseases will occur when the immune response inflicts damage to tissues in the body. Attempts to overcome this disease have a lot to do but they have not yielded satisfying results. In this study, the function of CD4⁺CD25⁺ regulatory T cells was evaluated for therapeutic potential to prevent the development of autoreactive T cells.

Methods: The isolation of CD4⁺CD25⁺ regulatory T cells was performed by using FACS vantage. It was conducted by taking a spleen of 6-week-old C57BL/6 mice. Cells having high purity (2x10⁶) were intravenously injected into 3-week-old CD122^{-/-} mice. In this experiment we used congenic mice as donors and transferred hosts. The result of adoptive transfer was examined after 7 weeks.

Result: Here we showed that the transfer of CD4⁺CD25⁺ regulatory T cells into CD122^{-/-} mice significantly prevented the development of abnormal T cells. The injection of CD4⁺CD25⁺ regulatory T cells derived from normal young mice into CD122^{-/-} mice could prevent the development of activated memory T cells and these cells were essential for maintaining normal homeostasis ($P < 0.01$).

Conclusion: In this study we obtained evidence that regulatory T cells (2x10⁶) derived from normal young mice could overcome diseases when transferred to the mouse models of autoimmune disorder.

Keywords: Regulatory T cells, autoimmune disease, autoreactive, CD4⁺CD25⁺.

INTRODUCTION

An autoimmune disorder is a condition occurring when the immune system mistakenly attacks and destroys healthy body tissue. These diseases are increased because of life style and genetic problem. One of the most important strategies to overcome the diseases is the use of regulatory T cells as therapy. Currently scientists classify regulatory T cells into two major groups, i.e., professional regulatory T cells and induced regulatory T cells [1-5]. Professional regulatory T cells emerge from thymus, whereas induced regulatory T cells emerges due to micro-environmental influence. Many types of regulatory T cells have been proposed, but CD4⁺CD25⁺ population is the most potent regulator which most researchers believe. In this study we show the importance of CD4⁺CD25⁺ regulatory as the component of immune system which can “cease-fire” between autoreactive cells in order not to damage tissue in the body. The immune system has evolved several mechanisms to control the expansion and differentiation of activated T cell, including energy, death, and regulation. Normally, immune system reactions are required to prevent a body against pathogen and tumor cells, but they can be harmful for an individual. Therefore, the immune system must be tightly regulated in order not to attack self-antigen [6, 7]. Currently it is known

that regulatory T cells play an important role in such regulation. CD4⁺CD25⁺ T cells are the most important part of T cells known and well-documented as a potent regulatory T cell [8-10]. For better understanding of autoimmune disorder, the mouse models of this disease have been engineered, namely CD122 knockout genes (CD122^{-/-}). CD122 (IL-2/IL-15 receptor β chain)-deficient mice are representative models of autoimmune disease with many symptoms such as gastritis, oophritis, orchitis, thyroiditis, pancreatitis, and colitis. CD122 (IL-2/IL-15 receptor β chain)-deficient mice exhibit severe hyper-immunity [11], with augmented granulopoiesis and suppressed erythropoiesis. Severe anemia progresses and the mice die within 3-4 months after birth. The generation mechanism of activated memory-type T cells in CD122^{-/-} mice is controversial. According to Rifa'i *et al.* [14], unhealthy condition in CD122^{-/-} is due to autoreactivity of T cells. In these mice most T cells become activated memory and very few exist in naïve phenotype. Abbas and others [12, 13], state that the programmed cell death (apoptosis) defect in IL-2^{-/-} or CD25^{-/-} T cells can be applied to the survival mechanism of activated IL-2R β -deficient T cells, and such possibility has not been excluded. Until now there has been no direct evidence for which population among CD4 and CD8 T cells responsible to regulate normal homeostasis. It is important to look for an explanation either the loss of the regulatory system may underlie the occurrence of reactivity T cells, or another necessary factor such defective expression of CD122 molecules is required. In this study, we show that injection of CD4⁺CD25⁺ regulatory cells derived from

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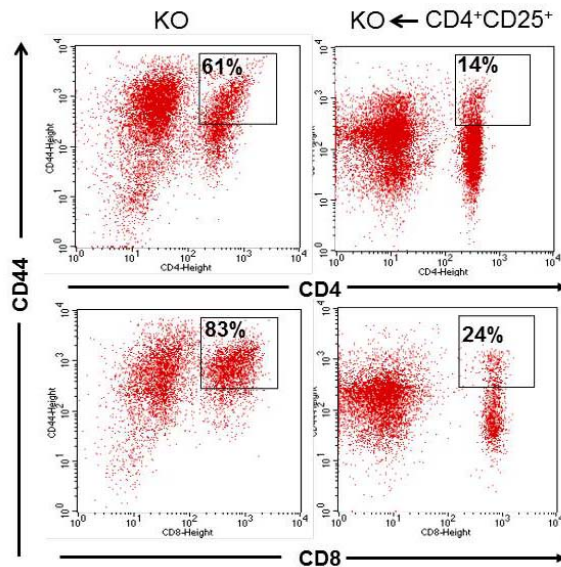


Fig. (1). CD122^{-/-} mice receiving CD4⁺CD25⁺ regulatory T cells at the age of 3 weeks did not develop autoreactive or activated memory type T cells. Spleen cells of CD122^{-/-} mice were analyzed 7 weeks after receiving 2x10⁶ regulatory T cells. Spleen cells were stained with anti-CD45.1, anti-CD8, anti-CD4 and anti-CD44 antibodies, and analyzed by flow cytometry. Host cells, both CD4 and CD8 T were presented with dot-plots as population gated to CD8⁺CD45⁻ or CD4⁺CD45.1⁺. Percentages of autoreactive or memory type T cells were shown in each panel with CD8⁺CD44⁺ or CD4⁺CD44⁺. Controls were CD122^{-/-} mice (KO) without manipulation. Data were mean of \pm SD values of five mice per group.

normal mice into 3-week-old CD122^{-/-} mice makes these hosts healthy and causes the phenotype to develop normally. This result indicates that CD4⁺CD25⁺ T cell population contains novel regulatory T cells that effectively control CD122-deficient T cells. The most important result is the fact that CD4⁺CD25⁺ regulatory T cells can ameliorate the condition of the mice that have been already sick for three weeks. This finding opens up opportunities to use regulatory T cells as therapeutic strategies for diseases involving cell activation. Since Rifa'i *et al.* [14] report that the usage of CD8⁺CD122⁺ regulatory T cells has a potential to inhibit autoimmune reaction in CD122-deficient mice when applied in neonate, it is important to combine these two regulatory T cells together.

MATERIALS AND METHODS

Animal

In this experiment we used C57BL/6^{-CD45.1/CD45.1} and CD122^{-/-} ^{-CD45.2/CD45.2} mice. They were housed in pathogen free facility. Regulatory T cells were isolated from C57BL/6-^{-CD45.1/CD45.1} normal mice, while the hosts were CD122^{-/-} ^{-CD45.2/CD45.2} mice.

Antibodies

Phycoerythrin (PE) or allophycocyanin (APCn)-conjugated anti-mouse CD4 (clone GK1.5), FITC- or biotin-conjugated anti-mouse CD45.1 (clone A20), Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD44 (clone IM7), (FITC)-conjugated anti-mouse CD8 α (clone 53-6.7), PE-conjugated anti-mouse CD62L (clone MEL-14), TER-119 (clone TER-119) antibodies were purchased from eBioscience Inc. (San Diego, CA). For FITC-conjugated anti-mouse CD69 (clone H1.2F3), biotin-conjugated anti-mouse CD122 (clone 5H4), FITC-conjugated anti-mouse CD25 (clone PC61.5), and FITC-conjugated anti-mouse Gr-1 (clone RB6-8C5), when we used biotin-conjugated antibodies, we visualized the antibodies by streptavidin-PE-Cy5 (eBioscience, San Diego, CA).

Flow Cytometry and Cell Sorting

To obtain highly purified CD4⁺CD25⁺ regulatory T cells we performed cell sorting by using FACS Vantage cell sorter (BD-Biosciences, San Jose, CA). Analytical flow cytometry was performed by using FACS CaliburTM flow cytometer (BD-Biosciences, San Jose, CA).

Hematocrit

Hematocrit was measured by taking peripheral blood from mice's tails, and heparin-coated capillary tubes were used to inhibit coagulation. The capillary tubes were sealed with clay, and centrifuged (3500 rpm) for 20 minutes. Then, the length of packed RBC per total blood was measured.

Statistical Analysis

One-way ANOVA was used to analyze the data. The differences between groups were considered significant at $P < 0.01$. All results were presented as the mean of \pm SD values of 5 mice in each group.

RESULTS

CD4⁺CD25⁺ regulatory T cells prevented the expansion of autoreactive and activated T cells. As shown in (Figs. 1-3), activated memory-type T cells in CD122^{-/-} mice would increase in the age of 7 weeks with markers of CD44⁺, CD62L⁻, and CD69⁺. In contrast, the same age CD122^{-/-} mice which received 2x10⁶ CD4⁺CD25⁺ cells did not show such increase of activated memory T cells. The transfer of 2x10⁶ CD4⁺CD25⁺ T cells to CD122^{-/-} mice did not prevent the accumulation of activated memory type T cells. We have data that CD4⁺CD25⁺ cells increase in the 2-year-old mice compared to those in 6-week-old ones. However, in this experiment we did not check whether CD4⁺CD25⁺ cells in old mice contained potent regulatory cell.

IL-2 receptor β chain (CD122) was essential for CD4⁺CD25⁺ development because host CD122-deficient mice which had been cured after receiving the transfer of CD4⁺CD25⁺ regulatory T cells had only donor type CD4⁺CD25⁺ regulatory T cells. It was very interesting that

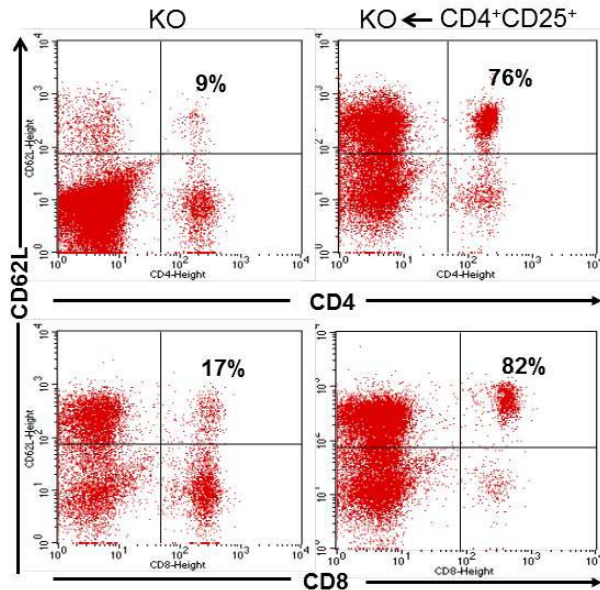


Fig. (2). CD122^{-/-} mice receiving CD4⁺CD25⁺ regulatory T cells at the age of 3- weeks developed naïve type T cells. Spleen cells of CD122^{-/-} mice were analyzed 7 weeks after receiving 2x10⁶ regulatory T cells. Spleen cells were stained with anti-CD45.1, anti-CD8, anti-CD4 and anti-CD62L antibodies, and analyzed by flow cytometry. Host cells, both CD4 and CD8 T were presented with dot-plots as population gated to CD8⁺CD45⁻ or CD4⁺CD45.1⁺. Percentages of naïve T cells were shown in each panel with CD8⁺CD62L⁺ or CD4⁺CD62L⁺. Controls were CD122^{-/-} mice (KO) without manipulation. Data were mean of ± SD values of five mice per group.

CD25 molecules could not be expressed by host even though the gene of the molecule was not deleted. It meant, genetical defect in CD122 gene affected CD25 gene product. The analysis using flow cytometer showed percentage of granulocytic Gr-1⁺ cells in CD122^{-/-} mice increased up to 82% in the bone marrow compared to about 55% in normal mice (data not shown). On the other hand, cells in erythroid lineage expressing TER-119 decreased in CD122^{-/-} mice. Most TER-119⁺ cells in the bone marrow were VLA4⁺ erythroblasts. These bone marrow changes affected the total condition of mice. Changes of TER-119 expression influenced the number of both white and red blood cells in peripheral blood. In CD122^{-/-} mice both CD4 and CD8 T cells increased and became activated, B cells dramatically decreased and almost missing from lymphoid tissue and circulatory (Fig. 4). The number of leukocytes markedly increased and hematocrit significantly decreased in CD122^{-/-} mice. These symptoms were signs of anemia progression in these mice. CD122^{-/-} mice (3 -weeks -old) injected with 2x10⁶ CD4⁺CD25⁺ cells had normal TER-119⁺/Gr-1⁺ cell ratio in the bone marrow, leukocyte number in peripheral blood, and hematocrit. On the contrary, CD122^{-/-} mice receiving 2x10⁶ CD4⁺CD25⁻ cells had TER-119⁺/Gr-1⁺ cell ratio skewed toward Gr-1⁺ cells, low hematocrit, and increased the number of activated T cells in peripheral blood (data not shown). In CD122^{-/-} mice, CD4⁺CD25⁺ regulatory T cells could not be induced from CD4⁺CD25⁻ cells, and the generation from bone marrow was also impaired. It meant CD4⁺CD25⁺ regulatory T

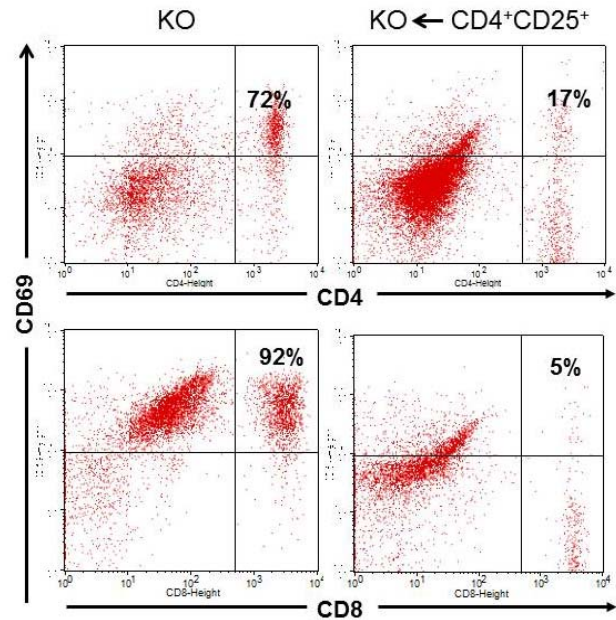


Fig. (3). CD122^{-/-} mice receiving CD4⁺CD25⁺ regulatory T cells at the age of 3- weeks did not develop early activated memory T cells. Spleen cells of CD122^{-/-} mice were analyzed 7 weeks after receiving 2x10⁶ regulatory T cells. Spleen cells were stained with anti-CD45.1, anti-CD8, anti-CD4 and anti-CD69 antibodies, and analyzed by flow cytometry. Host cells, both CD4 and CD8 T, were presented with dot-plots as population gated to CD8⁺CD45⁻ or CD4⁺CD45.1⁺. Percentages of early activated memory type T cells were shown in each panel with CD8⁺CD69⁺ or CD4⁺CD69⁺. Controls were CD122^{-/-} mice (KO) without manipulation. Data were mean of ± SD values of five mice per group.

cells had specific lineage different from another clone. In this case IL-2 signaling was suggested to play pivotal role for CD4⁺CD25⁺ regulatory T cell development, and the absence of IL-2 receptor or this cytokine would affect the development of CD4⁺CD25⁺ regulatory cell.

The experiment results described that CD4⁺CD25⁺ regulatory T cells controlled CD122^{-/-} T cells, so that normal homeostasis could be maintained. We proposed that autoimmune disorders in CD122^{-/-} mice were site effects of cell activation. In normal conditions, vertebrate activates immune system to overcome foreign antigen or invader. The best examples of foreign antigen or invader are viruses, bacteria and their product, cancer cell, and tissue or blood from donor. Antibodies will be produced by B cells to eliminate these foreign antigens which are notably harmful substances. In these mouse models of autoimmune disorder, B cell could not develop (Fig. 4). Consequently, the immunity related to the B cells also became impaired, that was why these mice had a short life in about 10-12 weeks.

CD4⁺CD25⁺ ameliorate erythropoiesis and B cell generation. To know the existent of erythropoiesis we examined the expression of TER-119 antigen as a specific marker of erythroid-lineage [15-17]. In this experiment we showed that the number of TER-119⁺ cells in the bone marrow of CD122^{-/-} decreased before the manipulation with regulatory T cells. This erythroid suppression could be rescued by the transfer

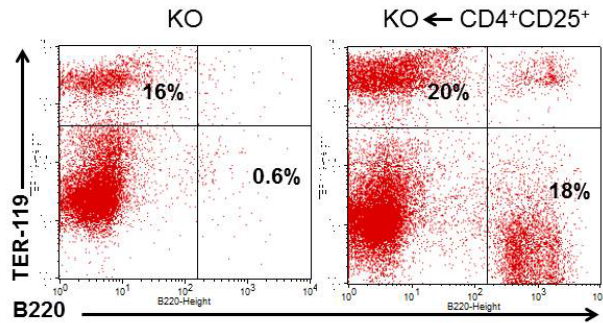


Fig. (4). B cells and erythrocytes normalization in $CD122^{-/-}$ mice that had received $CD4^{+}CD25^{+}$ regulatory T cells. Bone marrow cells of $CD122^{-/-}$ mice were analyzed 7 weeks after receiving 2×10^6 regulatory T cells. Bone marrow cells were stained with anti-CD45.1, anti-TER-119 and anti-B220 antibodies, and analyzed by flow cytometry. B lineage cells ($B220^{+}$) and erythroid lineage cells ($TER-119^{+}$) were presented with dot-plots as population gated to $B220^{+}CD45^{-}$ or $TER119^{+}CD45.1^{-}$. Controls were $CD122^{-/-}$ mice (KO) without manipulation. Data were mean of \pm SD values of five mice per group.

of 2×10^6 $CD4^{+}CD25^{+}$ cells into 3-week-old mice, whereas the transfer of 2×10^6 $CD4^{+}CD25^{-}$ conventional cells was not effective, and still showed the same decrease of $TER-119^{+}$ cells as that in non-manipulated $CD122^{-/-}$ mice. $CD4^{+}CD25^{+}$ regulatory T cells seemed to be the best candidate for autoimmune therapy since this cells had major vast of array in the context of immune system. In this experiment we clearly showed that the transfer with $CD4^{+}CD25^{+}$ T cells could prevent anemia when we examined hematocrit composition in peripheral blood. In contrast, an injection of conventional $CD4^{+}CD25^{-}$ cells with the same number of (2×10^6) did not ameliorate anemia condition in $CD122^{-/-}$ mice. In this experiment we observed the power of $CD4^{+}CD25^{+}$ regulatory T cells when these cells were adoptively transferred to $CD122^{-/-}$ mice. The transfer of $CD4^{+}CD25^{+}$ T cells (2×10^6) could normalize B cells development reflected by the increasing number of $B220^{+}$ cells, instead of 2×10^6 $CD4^{+}CD25^{-}$. The general condition of $CD122^{-/-}$ mice receiving 2×10^6 $CD4^{+}CD25^{-}$ did not change when compared to non-treated ones, and they all died before 12 weeks of age. It meant the regulatory T cells only occupied $CD4^{+}CD25^{+}$ population and could not be induced from $CD4^{+}CD25^{-}$ population. Many researchers believe $CD4^{+}CD25^{+}$ regulatory T cell is thymus origin and needs IL-2 signal to develop as functionally full competent cells.

In $CD122^{-/-}$ mice, granulocytic Gr-1⁺ cells were accumulated in bone marrow compartment. This accumulation was normalized when 3-week-old mice were transferred with 2×10^6 $CD4^{+}CD25^{+}$ cells, instead of $CD4^{+}CD25^{-}$ cells, in the same number. The measurement of leukocyte numbers in peripheral blood indicated that the increasing number of granulocytic Gr-1⁺ cells in bone marrow was closely related to that of leukocytes in peripheral blood. The increase of granulocytic Gr-1⁺ cells in bone marrow compartment and peripheral blood of $CD122^{-/-}$ mice could be one of compensations when the number of red blood cell decreased. By transferring 2×10^6 $CD4^{+}CD25^{+}$ regulatory T cells to 3-week-old mice, instead of transferring 2×10^6 $CD4^{+}CD25^{-}$ cells, the

leukocyte number in peripheral blood of $CD122^{-/-}$ mice could be normalized. Peculiar feature of $CD122^{-/-}$ mice could be one of thymic atrophy manifestations. The thymic atrophy had a very broad impact, including lack regulatory of T cells and abnormal development of activated memory type cells.

DISCUSSION

One of the most exclusive features of $CD4^{+}CD25^{+}$ cells is its capability to change the nature of already activated T cells, at least in the case of $CD122^{-/-}$ mouse-derived T cells. This fact could be seen in rescued 3-week-old $CD122^{-/-}$ mice normalized after receiving $CD4^{+}CD25^{+}$ regulatory T cells. It meant that $CD4^{+}CD25^{+}$ regulatory T cells had capacity to rescue autoimmune attack even though the component of individual immune system had been activated. The mechanism in which $CD4^{+}CD25^{+}$ regulatory T cells “ceased fire” among activated and aberrant cells was still illusive. In the age of 3- weeks, $CD122^{-/-}$ mice usually are developed with activated memory T cells and autoimmune symptom. However, transferred $CD4^{+}CD25^{+}$ regulatory T cells isolated from normal mice could reverse activated memory to naïve type. In this case CD44 and CD69 molecule were suppressed whereas CD62L was increased. After transferring with $CD4^{+}CD25^{+}$ regulatory T cells, CD44 and CD69 decrease significantly, it means that the status of the cells change from activated to naïve type. On the other hand, CD62L molecule expression increased after transferring with $CD4^{+}CD25^{+}$ regulatory T cells. CD62L was expressed in high level on naïve type and this molecule was lost in activated or memory type T cells. When T cells in the body were dominated by naïve type, they were likely healthy individuals. The capability of $CD4^{+}CD25^{+}$ regulatory T cells to reverse autoreactive cells could be related with production of IL-10 and TGF- β by these cells. There is a report that interposition of APC is required by $CD4^{+}CD25^{+}$ regulatory T cells in order for the cell to function effectively [18-21]. In this experiment we did not discriminate whether APC was involved in this system, but most probably APC did an intervention within *in vivo* system.

In studies by Malek *et al.* [22, 23], the defect of $CD4^{+}CD25^{+}$ regulatory T cells is proposed to be responsible for the increase of deregulated T cells in $CD122^{-/-}$ mice. It is based on the fact showing very few presence of $CD4^{+}CD25^{+}$ T cell in $CD122^{-/-}$ mice. The result of Malek *et al.* is consistent with our observation that $CD122^{-/-}$ mice develop autoreactive cells causing death for mice in about 3 months old. $CD4^{+}CD25^{+}$ regulatory T cells play important role to control autoreactive cells expressing high level of CD69 and CD44 molecules and loss of CD62L molecule on their cell surface. The absent of $CD4^{+}CD25^{+}$ cells initiated autoimmune reaction due to the loss of regulatory function. Although the precise mechanism of $CD4^{+}CD25^{+}$ cell action remains obscure at present, but the functionally important of this cells should be a concern for researchers. Interestingly, most $CD4^{+}CD25^{+}$ regulatory T cells in young mice expressed FOXP3 molecule, that was why purified $CD4^{+}CD25^{+}$ regulatory T cells from young mice were actually the same cell as $CD4^{+}CD25^{+}FOXP3^{+}$ T cells (data not shown).

CONCLUSION

This study concludes that there are regulatory T cells in normal individual. If these cells are lost from the body, autoimmune reactions will occur. These autoimmune reactions can be relieved by transferring CD4⁺CD25⁺ regulatory T cells derived from normal individuals, instead of transferring conventional CD4⁺CD25⁻ cells. CD4⁺CD25⁺ regulatory T cells work as a potent regulator by suppressing activated and autoreactive T cell and these cells can also normalize the general condition including the number of B cells. Also, they can increase red blood cells lineage (TER119), as in CD122^{-/-} which is deeply decreased. As the conclusive result, CD122-deficient mice receiving CD4⁺CD25⁺ regulatory T cells at the age of 3- weeks grow normally and remain healthy. Together this study explains that CD4⁺CD25⁺ regulatory T cells play pivotal roles to normalize homeostasis.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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ABBREVIATIONS

APC	=	antigen presenting cells
FITC	=	fluorescent iso-thiocyanate
PE	=	phycoerythrin

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