Comment on "CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes"

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Comment on "CCR7 Is Critically Important for Migration of Dendritic Cells in Intestinal Lamina Propria to Mesenteric Lymph Nodes"

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LETTERS TO THE EDITOR

Comment on "CCR7 Is Critically Important for Migration of Dendritic Cells in Intestinal Lamina Propria to Mesenteric Lymph Nodes"

The recent paper by Jang et al. (1), highlighted in *In This Issue* (2), is part of a recent rediscovery of the myeloid compartment of the intestinal lamina propria, which is probably the largest macrophage population in the body. The authors refer to these cells as dendritic cells, based on their expression of surface markers such as CD11c, but a dendritic cell was originally defined as a cell that can present Ag to naive T cells. The large majority of the stellate (or dendritic) cells of the intestinal lamina propria expresses the macrophage-specific F4/80 Ag, which is lost from mature Ag-representing cells, and also expresses the CSF-1 receptor and a CSF-1R-EGFP reporter gene (3) (see images of F4/80 and CSF-1R online ⟨www.macrophages.com⟩). The majority of the so-called dendritic cells of the lamina propria is clearly phagocytic (1). Some 15 years ago, the myeloid cells of mouse lamina propria were isolated, and the cells that could actually stimulate naive T cells in a MLR were selectively enriched in the nonphagocytic population (4). By contrast, the F4/80-positive phagocytes, which were also strongly class II-MHC-positive, caused indomethacin-sensitive repression of T cell activation. It is inescapable that the large majority of the cells studied by Jang et al. (1) are not dendritic cells by the functional definition, and there is no evidence that all, or indeed any, of them ever become cells that can present Ag to naive T cells. They are the lamina propria macrophages. They might acquire Ag-presenting activity. On the other hand, they might actually be more important to the maintenance of tolerance.

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Response to Comment on "CCR7 Is Critically Important for Migration of Dendritic Cells in Intestinal Lamina Propria to Mesenteric Lymph Nodes"

In response to a letter by Dr. David Hume (1), we would like to point out that the following lines of evidence clearly indicate that the lamina propria dendritic cells (LP-DC) we reported (2) are indeed DC but not macrophages. As seen in Fig. 1*A* in our recent analysis in which we used a 17.5% Accudenz solution instead of the original 15.5% solution to enrich for light-density cells such as macrophages and DC from the small intestinal LP, $CD11c^{high}CD11b^{low}$ (R1) and $CD11c^{high}CD11b^{high}$ (R2) cell populations with dendritic morphology were abundantly found and were strongly positive for a DC-specific marker, DEC-205. With regard to F4/80 expression, R1 subset was clearly negative, but R2 was moderately positive. Functional analysis revealed that both R1 and R2 subsets can present alloantigen to naive T cells just as effectively as splenic DC and lymph node DC (Fig. 1*B*, *left column*) and can also present OVA efficiently to OVA-TCR expressing T cells (Fig. 1*B*, *right column*). Thus, these $DEC\text{-}205^{+}$ LP-DC have the hallmark of DC, i.e., potent Ag-presenting abilities to T cells.

Then, are the intestinal F4/80-positive, MHC class II-positive phagocytic macrophages described by Dr. Hume's group (3) also found in our analysis? The answer seems yes. They appear to represent $CD11c^{int}CD11b^{high}$ cell population (R3) because they are strongly F4/80 positive, DEC-205 negative (Fig. 1*A*), and also strongly positive for MHC class II expression (data not shown). Although this cell population was hardly detectable in our original analysis (2), the use of 17.5% Accudenz solution instead of 15.5% solution in the present series of analysis allowed us to visualize such cells.

Collectively, by functional definition, LP-DC (2) are indeed DC with potent Ag-presenting abilities. Dr. Hume's finding (3) is also correct that macrophages are present in substantial numbers in the small intestinal LP, but they appear clearly distinguishable from the LP-DC (2).

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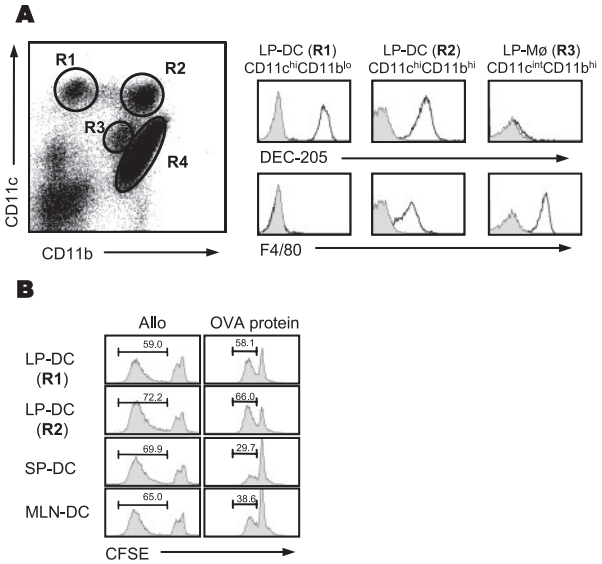

Figure 1. CD11c^{high} subsets in the small intestinal LP are DC but not macrophages. *A*, Low-density lamina propria cells were isolated from the BALB/c small intestine using a 17.5% Accudenz gradient. Enriched cells were stained for CD11b, CD11c, DEC-205, and F4/80 and analyzed by flow cytometry. Several CD11c^{+} subsets could be recognized on the basis of their different CD11c/CD11b expression patterns: CD11c^{high}CD11b^{low} (R1), CD11c^{high}CD11b^{high} (R2), CD11c^{int}CD11b^{high} (R3), and CD11c^{low}CD11b^{high} (R4; consisting of mainly eosinophils). Histogram FACS profiles shown on the *right* were acquired after gating on the R1, R2, and R3 subsets. Shaded, isotype controls; open, stained for DEC-205 or F4/80. *B*, LP-DC were examined for their Ag-presenting abilities. FACS-sorted BALB/c LP-DC were either cocultured with CFSE-labeled C57BL/6 spleen cells for 3 days (*left column*) or pulsed with OVA protein first and then cocultured with CFSE-labeled OVA TCR transgenic CD4^{+} T cells (DO11.10; 1:20) for 3 days (*right column*). DC from other tissues were also treated in the same way. T cell proliferation was measured by CFSE dilution. Numbers within histograms indicate the percentage of proliferated cells.

Comment on "Cutting Edge: Anti-CD25 Monoclonal Antibody Injection Results in the Functional Inactivation, Not Depletion, of CD4^{+}CD25^{+} T Regulatory Cells"

In a recent article, Kohm et al. (1) make a case for the in vivo administration of anti-CD25 leading to a functional inactivation of CD4^{+}CD25^{+} regulatory T cell (Treg), rather than their deletion as has been previously assumed. The majority of the data in the Kohm et al. article comes from the use of the 7D4 (rat IgM) Ab. We believe it is important to emphasize that, overwhelmingly, "Treg depletion" studies use a different clone, PC61 (rat IgG1). Kohm et al. suggest that down-modulation of CD25 after Ab treatment could lead to a misinterpretation that CD25-expressing cells are removed. This may be the case when using 7D4. However, using FACS analysis at the single cell level, we have previously reported that PC61 administration leads to a rapid and dramatic loss of CD25^{+}Foxp3^{+} cells without a corresponding increase in CD25^{-}Foxp3^{+} cells (2). These data lead inevitably to the conclusion that the commonly used PC61 Ab does, in fact, deplete CD25-expressing Treg.

So we are left with the interesting situation of two anti-CD25 Abs having very different effects. This is perhaps not surprising given their difference in isotype. These differences seem to be reflected in disease. Kohm et al. have previously reported that 7D4 does not influence the course of experimental autoimmune encephalomyelitis (EAE) after optimal immune priming (3, 4), whereas their most recent data do show exacerbation when priming is suboptimal (1). In contrast, we and others have found PC61 treatment to exacerbate/prolong EAE after both optimal and suboptimal priming (2, 5–7). As a whole, these studies indicate that Treg do have a controlling influence over optimal disease, and their removal using PC61 allows for an increase in severity and/or failure to recover. 7D4 appears not to achieve this, but perhaps perturbs the Treg/T-aggressor balance sufficiently to allow the conversion of silent autoimmunity into overt autoaggression in the setting of suboptimal priming. It will be important to dissect the mechanisms underlying disease modification in both these settings.

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Comment on "Cutting Edge: Anti-CD25 Monoclonal Antibody Injection Results in the Functional Inactivation, Not Depletion, of CD4^{+}CD25^{+} T Regulatory Cells"

The injection of anti-CD25 mAb to deplete CD25^{+} regulatory T cells (T_R) in mice has been common practice to infer the function of T_R. In a recent report, Kohm et al. (1) claimed that injection of anti-CD25 mAb in mice results in the functional inactivation, not depletion, of CD4^{+}CD25^{+} T_R. This conclusion was based on the analysis of mice that received the rat-IgM 7D4 mAb or the rat IgG PC61. CD25 and FOXP3 expression was determined by FACS analysis after 7D4 treatment but solely by RT-PCR after PC61 administration

(1). Using FACS analysis in both cases, we enumerated $CD4^+CD25^+$ and $CD4^+FOXP3^+$ cells in blood, spleen, and lymph nodes isolated from mice treated with either 7D4 or PC61 (Fig. 1). Similarly to Kohm et al., we found that despite the disappearance of surface CD25 expression, $FOXP3^+$ cell frequency is not affected by 7D4 administration, irrespective of the tissue analyzed and independently of the dose used (up to 3 mg/mouse). In contrast, injection of as little as 500 μg/mouse of PC61 resulted in both the disappearance of $CD25^+$ cells and the loss of $CD4^+FOXP3^+$ cells. In PBL, the frequency of CD4 cells expressing FOXP3 is reduced from an average of 8 to 3%, indicating that >65% of the total $FOXP3^+$ cells are depleted. The disappearing cells are the bulk of the $FOXP3^+CD25^+$ T_R, while the pre-existing $FOXP3^+CD25^-$ subset appeared maintained. In spleen, ~45% of the $FOXP3^+$ cells were depleted. T_R originally $CD25^+$ that lost CD25 expression and pre-existing $CD25^-$ T_R appeared to contribute equally to the remaining $FOXP3^+$ cell pool. In lymph nodes, depletion was less marked and corresponded to the disappearance of 33% of the $FOXP3^+CD25^+$. Increasing the amount of PC61 injected (up to 3 mg/mouse) did not dramatically increase the depletion efficiency (data not shown). We conclude that injection of the rat-IgG PC61 mAb, which has been the most frequently used to interfere with T_R function in vivo, does result in depletion of a large fraction of T_R.

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Response to Comment on "Cutting Edge: Anti-CD25 Monoclonal Antibody Injection Results in the Functional Inactivation, Not Depletion, of $CD4^+CD25^+$ T Regulatory Cells"

It is indeed important to dissect the mechanisms by which injection of anti-CD25 mAb may influence both $CD4^+CD25^+$ regulatory T cell (T_R) function and responder T cell functionality in vivo. This important need was the motivation of the current studies. While our current study does focus on the use of the 7D4 clone, we have observed similar findings using the PC61 clone (see Fig. 3 of Ref. 1). However, differences do exist.

Basic immunology tells us that an Ab of the isotype of IgM should be more efficient in fixing complement, and hence, serve to more efficiently deplete a target cell population. Interestingly, a previous study reported that neither PC61 nor 7D4 "induced a strong complement-mediated cytotoxicity" (2). We have tested this ourselves and have found that 7D4, but not PC61, fixes complement in vitro. Thus, we are left with the question that if PC61 indeed cannot fix complement, then by what mechanism does it deplete $CD4^+CD25^+$ T_R? Based on the capacity of fixing complement, it would seem that if either Ab were to be successful in depleting a target cell population, it would be the clone capable of fixing complement, i.e., 7D4. In vitro, as determined by triple staining of target T cells to measure the expression of CD4, CD25, and intracellular Foxp3, we have observed that CD25 expression is quickly down-regulated following exposure to either 7D4 or PC61 in the absence of cell depletion. In vivo, a fundamental difference between these two clones appears as revealed by the kinetics of CD25 expression, which is quickly down-regulated following in vivo treatment with 7D4. In comparison, the kinetics of PC61-induced CD25

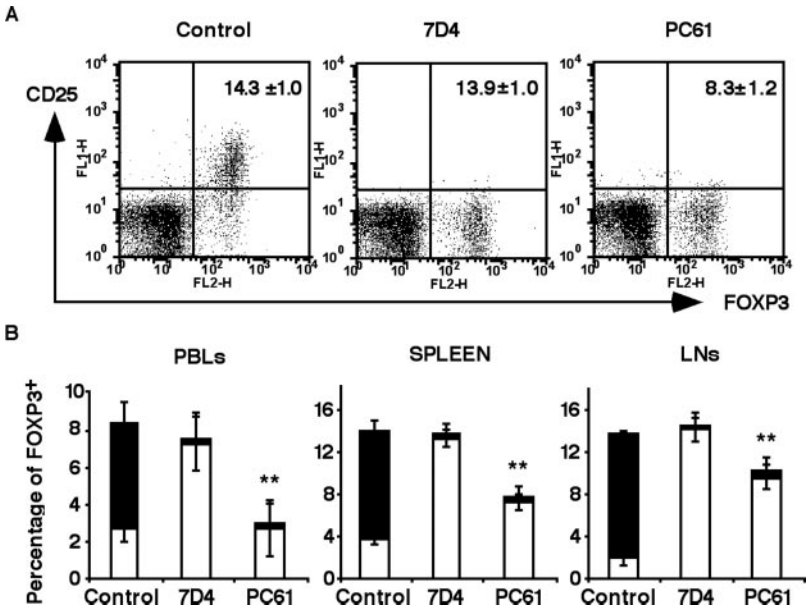

Figure 1. C57BL/6 mice (8 wk old) raised in specific pathogen-free conditions were injected i.p. once with anti-CD25 mAb clone 7D4 (3 mg) or PC61 (500 μg). Splenocytes, lymph node cells, and PBL were isolated 3 days after from either control or treated mice and analyzed by flow cytometry for CD4, FOXP3, and CD25 expression (for CD25 visualization, clone 7D4 or PC61 was used when PC61 or 7D4 was injected, respectively). *A*, Representative staining of splenocytes (gated on $CD4^+$ cells) analyzed for CD25 and FOXP3 expression. Number inside dot plots represents average ± SD of the percentage of $FOXP3^+$ among $CD4^+$ cells. *B*, Percentage of $FOXP3^+CD25^+$ (■) and $FOXP3^+CD25^-$ (□) among $CD4^+$ cells. **, $p < 0.01$ for $CD4^+FOXP3^+$ in PC61-injected mice vs control or 7D4-injected mice. Data represent the average ± SD for each group ($n = 3$). One of three independent experiments is shown.

down-regulation are slightly delayed; however, the effects appear to be longer-lasting than that of 7D4. Regardless of which isotype is used, we often see that CD25 expression returns to control levels within 7–10 days of the last treatment. It was this finding that originally prompted us to investigate the alternative actions of anti-CD25 mAb treatment, as it was hard to envision how the $CD4^+CD25^+$ T_R population could return to control levels within that time frame if the cells were physically depleted.

We are still investigating whether injection of PC61 results in a partial depletion of $CD4^+CD25^+$ T_R and the potential contribution that partial depletion may make to the differences in CD25 expression kinetics between the two Ab clones. While we cannot comment on the purported depletion of $CD4^+CD25^+$ T_R referenced by our colleagues (3), because Stephens and Anderton used a single dose of anti-CD25 mAb at a concentration double than we used in our studies, this difference is an obvious source for potential differences in the outcome of anti-CD25 mAb treatment. Furthermore, Zelenay and Demengeot (4) only observed partial deletion of $CD4^+CD25^+$ T_R using PC61. Our studies have revealed that decreasing the dose of anti-CD25 mAb treatment does decrease the duration of CD25 down-regulation.

In regards to the findings of McGeachy et al. (5) concerning the effects of PC61 on experimental autoimmune encephalomyelitis progression in disease induced under "optimal" priming conditions, it would be inappropriate for us to compare our findings to their report in light of the fact that the two model systems use distinct strains of mice, priming Ags, and priming protocols and that significant strain differences exist in regards to the functionality of $CD4^+CD25^+$ T_R. In sum, our data using clone 7D4 clearly demonstrate that CD25 down-regulation, in the absence of overt CD25 T_R depletion (as confirmed by Zelenay and Demengeot, Ref. 4), is capable of inactivating $CD4^+CD25^+$ T_R function allowing exacerbation autoreactive Th1 cell function and clinical experimental autoimmune encephalomyelitis progression.

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Comment on "Duration and Intensity of NF-κB Activity Determine the Severity of Endotoxin-Induced Acute Lung Injury"

Everhart et al. (1) reported that endotoxin-induced lung edema was completely prevented by NF-κB inhibition and incompletely by neutrophil depletion. Factors other than pulmonary neutrophilia were also involved in the pathogenesis of endotoxin-induced pulmonary edema, which the authors stated are currently not well defined. Some of these factors are well defined and are the cytokines TNF, IL-1, and IFN-γ and their mediator NO. These cytokines contribute to pulmonary edema by a reduction of alveolar sodium and chloride and associated fluid transport (2). TNF reduced epithelial sodium channel mRNA expression in alveolar epithelial cells (3). IL-1 was noted to reduce cystic fibrosis transmembrane conductance regulator chloride channel function and epithelial sodium channel expression in alveolar type II cells. IFN-γ reduced cystic fibrosis transmembrane conductance regulator mRNA in respiratory epithelial cells (2). In meningococcal septicemia, pulmonary edema was associated with reduced epithelial chloride transport (4).

NO is released by distal lung epithelial cells and alveolar macrophages in response to TNF, IL-1, and IFN-γ (2) and reduced sodium transport in alveolar epithelial cells (5). Neutrophil leukocytes are a source of both TNF and IL-1. The lung edema in neutrophil depleted mice was probably induced by these mediators generated by cells other than neutrophils like local macrophages. Future research into the effects of NF-κB inhibition on pulmonary edema needs to focus on TNF, IL-1, IFN-γ, and NO production and the associated changes in alveolar ion and fluid transport.

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