LETTERS TO THE EDITOR

Response to Comment on "Cathepsin-L Influences the Expression of Extracellular Matrix in Lymphoid Organs and Plays a Role in the Regulation of Thymic Output and of Peripheral T Cell Number"

In our article published in *The Journal of Immunology* in June 1, 2005 (1), we reported that lymph nodes from *nackt* mice (*CTSL*^{nkt}/*CTSL*^{nkt}) are hypertrophied, showing a normal absolute number of CD4$^+$ T cells and a marked increase in the number of CD8$^+$ T lymphocytes. Correlatively, extracellular matrix (ECM) components were found to be increased. Contrarily, in *nackt* thymus, laminin, fibronectin, and collagen I and IV are markedly decreased, with an augmented output of CD8$^+$ cells. We also reported that a mutated form of cathepsin L can be detected in different organs in *nackt* mice. These results demonstrate that the *nackt* mutation in the *Ctsl* gene influences the levels of ECM components in lymphoid organs, the thymic output, and the number of T cells in the periphery, thus broadly affecting the immune system.

In a letter to the editor, Benavides et al. (2) show that it is possible to detect a mutated cathepsin-L (CTSL) in *nackt* mice, indeed confirming our results. As stated in our article, it is clear that the *nkt* mutation produces a mutated CTSL devoid of its classical proteolytic activity. The 118-bp deletion of *nkt* mice (2) involves the end of exon 6 and almost all of exon 7, sequences partially coding for the protein H and L chains. As a consequence of this deletion, a stop codon would appear leading to a truncated peptide lacking the last 60 aa, which are included in the active site of this protease (3). However, they claim that the *nackt* allele clearly behaves as a recessive loss-of-function mutation where heterozygous mice are phenotypically indistinguishable from wild-type mice and homozygous mutants exhibit the same phenotype as *Ctsl* knockout (KO) mice. Regarding the differences between wild-type and +/*nkt* mice, Benavides et al. (2) have reported that the skin of +/*nkt* mice shows no major difference with that of +/+ mice. However, in our hands, significant differences in the level of expression of integrins between +/+ and +/*nkt* mice can be detected (Fig. 1; I. Piazzon and I. Nepomnaschy, manuscript in preparation). As an example, data on Fig. 1 show that the percentage of lymph node CD4$^+$ cells expressing high levels of α_6 integrin chain is 2-fold increased in +/*nkt* vs +/+ mice. Thus, on one hand, it cannot be discarded that haploinsufficiency affecting different traits can be detected in +/*nkt* mice, as it has been described for the skin and the trabecular bone volume of *CTSL* KO mice (B6; 129-*Ctsl*^{tm1Alpk}) (4). On the other hand, we have suggested the possibility that the presence of a mutated CTSL devoid of proteolytic activity in *nackt* mice could lead to the appearance of phenotypic differences between *CTSL* KO and *nackt* mice. It is unclear whether the *nackt* phenotypes we have described (1) are shared by the *CTSL* KO mice. The total cellularity of spleen and thymus in wild-type and *CTSl*^{tm1Cptr}/*CTSl*^{tm1Cptr} mice has been reported to be comparable (5), and *nackt* mice also show normal number of thymocytes and splenocytes. It has not been reported that lymph node cellularity is altered in *CTSL* KO mice, while *nackt* mice show increases in the number of lymph node cells. Nakagawa et al. (5) had shown that the percentage of CD8$^+$ thymocytes is increased in *CTSl*^{tm1Cptr}/*CTSl*^{tm1Cptr} mice, while no differences could be detected in *nackt* mice. To our knowledge, the additional phenotypes, including differences in extracellular matrix components in the thymus and lymph nodes that we have described, have not been investigated so far for *CTSL* KO mice. Chauhan et al. (6) had suggested that human *CTSL* lacking the 16 C-terminal amino acids is retained in the endoplasmic reticulum presumably because of its improper folding. The possibility exists that the presence of a mutated CTSL in *nackt* mice could lead to the appearance of phenotypic differences between KO and *nackt* mice. Direct testing of this hypothesis would be required.

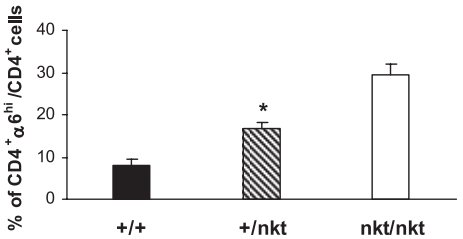

Figure 1. α_6 integrin chain expression in CD4$^+$ lymph node cells of +/+, +/*nkt*, and *nkt/nkt* mice. Lymph node cells from +/+, +/*nkt*, and *nkt/nkt* littermates were stained with anti-CD4 and anti-CD49e and analyzed by flow cytometry. Values represent the mean percentages of cells expressing high levels of α_6 integrin chain ± SD ($n = 6$) within CD4$^+$ cells. *, Significantly different from +/+ ($p < 0.01$); from *nkt/nkt* ($p < 0.01$).

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Comment on "Analysis of the Cellular Mechanism of Antitumor Responses and Autoimmunity in Patients Treated with CTLA-4 Blockade"

In the December 1, 2005, issue of *The Journal of Immunology*, Maker et al. (1) conclude that the effects of CTLA-4 blockade are due to increased T cell activation rather than inhibition or depletion of T regulatory cells. The authors examined the effects of anti-CTLA-4 administration on T regulatory cells before and at 3 wk after each dose. They saw no reduction in $CD4^+CD25^+$ T cells, and to characterize further the T regulatory cell population, they examined *Foxp3* gene expression. Increased *Foxp3* expression was observed at 3 wk posttreatment compared with pretreatment levels in purified $CD4^+CD25^+$cells. We believe their conclusion that T regulatory cell depletion is not involved in the mechanism of action of anti-CTLA-4 is flawed by a failure to examine its effect at early time points after administration. We postulated that expression of CTLA-4 on T regulatory cells targeted them for Ab-dependent cytotoxicity. PBMCs from patients with advanced malignancy were examined before, within 1–4 days, and 3–4 wk after anti-CTLA-4 administration. At 1–4 days after administration, there was a significant decrease in the number of T regulatory cells as quantitated by expression of CD4, CD25, and CD62L. However, at the time of administration of the next dose, the number of T regulatory cells had increased above baseline, in agreement with the results of Maker et al. These results were confirmed by TaqMan analysis for *Foxp3* expression; a decrease at early times was followed by a rebound increase by the next treatment. This depletion followed by increased levels of *Foxp3* assayed by TaqMan mirrored the results from flow cytometry. The dynamic changes in T regulatory cells was missed in Maker's analysis by restricting data collection time points. We agree, but have been unable to demonstrate, that increased T cell activation is responsible for the antitumor effects of anti-CTLA-4 but believe that transient depletion of T regulatory cells permits activation of T cells that recognize self Ags, which in turn produce autoimmunity and tumor regression.

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Response to Comment on "Analysis of the Cellular Mechanism of Antitumor Responses and Autoimmunity in Patients Treated with CTLA-4 Blockade"

In our article analyzing the cellular mechanisms of antitumor and autoimmune effects of CTLA-4 blockade (1), the cells that were tested were obtained ~3 wk after the last dose of anti-CTLA-4 mAb. All patients had received many doses of anti-CTLA-4 Ab administered every 3 wk, and because the half-life of this Ab is 2–3 wk, it would be expected that any depletion of T regulatory cells would be seen at the 3-wk time point. It is possible, however, as Drs. O'Mahony and Janik point out, that we could have missed a decrease in T regulatory cells at earlier time points. In fact, Drs. O'Mahony and Janik point out that at 1–4 days after administration of anti-CTLA-4 Ab they did see a significant decrease in the number of T regulatory cells.

As a result of their letter, we have performed additional experiments testing the presence of T regulatory cells obtained before and 4 days after administration of anti-CTLA-4 mAb in a patient in our protocol. This patient had received 5 mg/kg anti-CTLA-4 Ab twice and 9 mg/kg twice every 3 wk. Before and 4 days after the fifth dose at 9 mg/kg, blood was obtained and cell analysis was performed simultaneously on these cryopreserved specimens. As in our prior results, there was no evidence of decrease in T regulatory cells. Comparing pre- and post-4-day treatment samples, the $CD4^+$*Foxp3*$^+$ cells increased from 14.6 to 18.2%, and the percentage of *Foxp3*$^+CD25^+$ cells increased from 23.0 to 34.4%. In addition, we purified $CD4^+$ cells and performed semiquantitative RT-PCR analysis of *Foxp3* levels in these cells. Following immunomagnetic bead purification, the CD4 cells isolated by negative selection were 90–94% pure. In samples obtained 4 days after administration of anti-CTLA-4 Ab, the number of *Foxp3* mRNA copies per 10^6 β-actin copies increased from 1419 to 1862.

Thus, the results we obtained in this patient studied 4 days after administration of anti-CTLA-4 Ab were similar to those obtained in samples obtained 3 wk following administration of the Ab. Although further studies on samples taken at short times after administration of anti-CTLA-4 Ab are warranted, it seems unlikely to us that the mechanism of action of anti-CTLA-4 Ab administration is due to a decrease of T regulatory cells.

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