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Letters to the Editor

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Comment on "Cutting Edge: Regulatory T Cells Do Not Mediate Suppression via Programmed Cell Death Pathways"

We read the article by Szymczak-Workman et al. (1) with great interest. They have concluded that apoptosis of effector CD4 T cells is not involved in $CD4^+Foxp3^+CD25^+$ regulatory T cell (Treg)-mediated suppression, despite several studies showing the pivotal role of Treg-mediated apoptosis in immune suppression (2–6). Since the authors' experimental conditions differed from our work (4), we tested different T cell activation conditions for Treg suppression (7). We found proliferative suppression consistently associated with apoptosis of T responder (Tresp) cells as previously reported (Fig. 1A, 1B) (4). Using the CD4 Tresp cells from Bim knockout (BKO) mice obtained from The

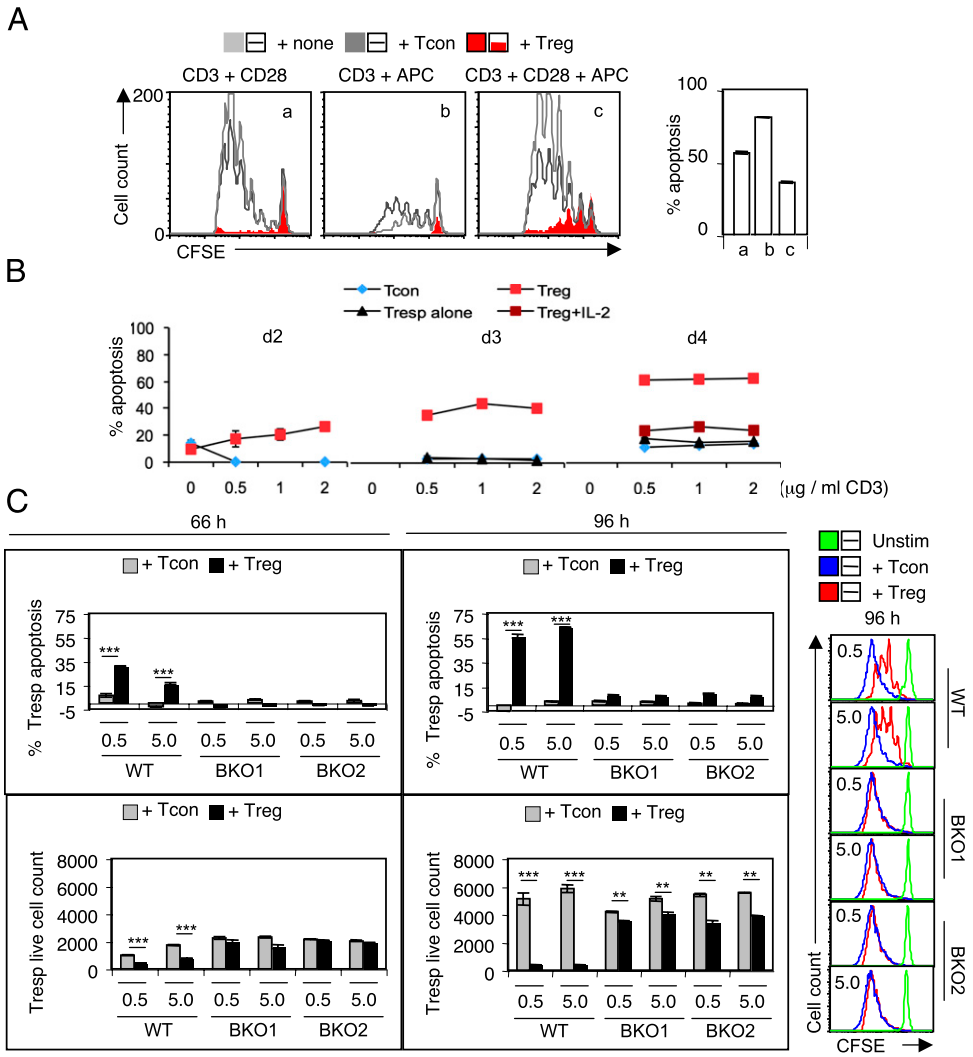


FIGURE 1. CD45.2 $CD4^+$ $CD25^-$ (Tresp), control CD45.1 $CD4^+$ $CD25^-$ (Tcon), and CD45.1 $CD4^+$ $CD25^+$ (Treg) cells were FACS sorted from spleen. Tresp cells were cultured alone or in cocultures with Tcons or Tregs at a 1:1 ratio. (**A**) The cells were stimulated using soluble anti-CD3 Ab (0.5 μg/ml) along with anti-CD28 Ab (2 μg/ml) (**a**), anti-CD3 Ab along with APC (**b**), or anti-CD3 Ab along with anti-CD28 Ab and APC (**c**). Live cell proliferation (*left*) and the percentage of Treg-induced apoptosis (*right*) of CFSE-labeled Tresp cells, as detected 4 d after culture. (**B**) The cells were stimulated using indicated concentrations of soluble anti-CD3 Ab and 2 μg/ml of anti-CD28. IL-2 was added where indicated. (**C**) The cells were stimulated using indicated concentrations (0.5 or 5 μg/ml) of soluble anti-CD3 Ab and 2 μg/ml of anti-CD28 and APC for 66 or 96 h. Percentage Tresp apoptosis (*upper panel*), CFSE + propidium iodide − Tresp live cell counts (*lower panel*) or proliferation (*right*) of CD45.2 Tresps, as measured by flow cytometry in equal volumes of cells and at constant times. $***p < 0.005$, $**p < 0.05$.

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Jackson Laboratory (Bar Harbor, ME) (BKO1) and from Vignali's group (BKO2) (8), we found that they underwent substantially less apoptosis consistent with protection against cytokine withdrawal apoptosis and were largely rescued from proliferative suppression, verifying our previous conclusion (4) (Fig. 1C). We observed modest decreases in cells on day 4 in Treg cocultures, which we attribute to a partial proliferative arrest due to growth cytokine consumption by the Tregs. The results verify that apoptosis of Tresp CD4 T cells clearly plays an important role in Treg suppression (4). There are several potential technical explanations for the Szymczak-Workman et al. observations (1), but we hesitate to speculate on why their conclusions could not be replicated in our hands. Notably, Vignali's group (8) has reported in the same issue of *The Journal of Immunology* that suppression by Tregs can be mediated by the apoptosis-inducing TNF family ligand TRAIL, which also appears to contradict the broad conclusion of the Szymczak-Workman et al. article (1).

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Response to Comment on "Cutting Edge: Regulatory T Cells Do Not Mediate Suppression via Programmed Cell Death Pathways"

We thank Drs. Pandiyan and Lenardo for their interest in our study (1). Defining the mechanisms used by T regulatory cells (Tregs) to mediate suppression is an important but contentious topic (2). Our study specifically asked if T cells resistant to apoptosis mediated by the proapoptotic molecule Bim could be suppressed by Tregs (1). This was stimulated by an earlier study in which Pandiyan, Lenardo, and colleagues (3) suggested that Tregs induce IL-2 deprivation-mediated apoptosis and that $Bim^{-/-}$ T cells were resistant to Treg-mediated suppression. The relative contribution of this mechanism remains controversial as more recent studies have also suggested that IL-2 depletion alone is not required for the suppression of human T cells (4, 5). While we appreciate the comments from Drs. Pandiyan and Lenardo on our study, we feel that they have overlooked some important considerations and misinterpreted some published observations.

First, our study provided extensive in vitro and in vivo analysis that clearly showed that Treg-mediated suppression of $Bim^{-/-}$, $Bim^{-/-}Puma^{-/-}$, and Bcl2 transgenic T cells is comparable to controls (1). While in vitro assays can be very informative, all too often strong conclusions are drawn from data obtained primarily from such assays. It is clear that the culture conditions used can dramatically affect the rate of T cell death observed following T cell activation. Although we are similarly reticent to speculate on why the conclusions of Pandiyan, Lenardo, and colleagues could not be replicated in our hands, it seems tenuous to rebut our observations with data obtained exclusively from in vitro assays. Furthermore, the data accompanying their comments above are derived from a single experiment using only four mutant mice. The primary finding reported in our study (1) was based on solid in vivo analysis and thus we would argue that any challenge to these observations should also be derived from in vivo studies.

Second, our study proposed that Tregs do not use Bim, Puma or Ripk3-mediated programmed cell death pathways as a dominant, broadly utilized mechanism of suppression. However, to be accurate, we did not claim that Tregs cannot mediate suppression by any cell death pathway under any circumstances. As clearly stated in our study ". . . we would argue that if Bim-mediated apoptosis contributed significantly to Treg function, it would have been revealed in this assay . . . Nevertheless, we cannot rule out the possibility that this mechanism is used by Tregs but only under unique circumstances or disease scenarios. Furthermore, although Bim and Puma are primary mediators of apoptosis, we cannot rule out the possibility that Tregs use mechanisms of cell death that are Bim, Puma, and Ripk3 independent and not blocked by Bcl-2" (1). So while Drs. Pandiyan and Lenardo suggest above that several studies have shown a ". . . pivotal role [for] Treg-mediated apoptosis in immune suppression," all of these studies, except theirs, focused on Bim-independent pathways of cell death.

Third, Drs. Pandiyan and Lenardo suggest that another study of ours (6) contradicts the study in question (1). This is incorrect. We clearly showed that TRAIL is not a major mechanism used by C57BL/6 Tregs, the population examined by Pandiyan, Lenardo, and colleagues (3), but is utilized by IL-10– and IL-35–deficient C57BL/6 Tregs or by wild type Balb/c Tregs.

In closing, while we differ in our interpretation of the data presented, it is clear that there are many unresolved issues related to Treg function, in particular the contribution of IL-2

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deprivation-mediated apoptosis, which require further examination.

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Comment on "Frequency of Epitope-Specific Naive CD4$^+$ T Cells Correlates with Immunodominance in the Human Memory Repertoire"

We read with interest the article by Kwok et al. (1), which reported the tetramer-guided epitope mapping of protective Ag (PA)-specific CD4$^+$ T cells from both anthrax-vaccine adsorbed vaccinees and naive individuals.

We have evidence that the three putative epitopes reported by the authors also form part of the T cell responses of naturally infected cutaneous anthrax patients (2). Previous studies identifying immunodominant PA epitopes in humans have concentrated upon the responses of vaccinated individuals (3, 4). It is therefore gratifying, despite the potential for repeated vaccinations to skew the T cell epitope repertoire, to discover parity between the responses of vaccinees and patients, suggesting that these immunodominant epitopes are efficiently processed and presented within the context of both vaccination and infection. Furthermore, while the authors (1) focused on the strong binding affinity of these epitopes to HLA-DR0101, we found that the peptides containing these epitopes also show strong affinity for a range of common HLA-DR alleles (Table I). These epitopes potentially play a part in the cellular immune responses of a wide section of the population.

The authors (1) suggest the immunodominance hierarchy may be related to the relative frequencies of PA-specific naive T cells. The possibility of using tetramer-guided epitope mapping in naive subjects to detect Ag-specific epitopes that are not only relevant following vaccination but also form a demonstrable part of the natural immune response to the live pathogen represents an interesting new avenue for determining immunodominant epitopes derived from organisms such as *Bacillus anthracis*.

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Table I. PA epitopes identified in the T cell response of naturally infected cutaneous anthrax patients show strong binding to a range of common HLA-DR alleles

PA Peptide	HLA Class II DR1	DR3	DR4	DR7	DR11	DR13	DR15
^{400}**TAPIYNVLPTTSLVLGKNQT**^{419}	**1**	**12**	**2**	**0.4**	**78**	300	**6**
^{510}**LPQIQETTARIIFNG**KDLNL^{529}	**2**	**5**	886	**0.3**	**37**	**2**	**1**
^{710}**YNDKLPLYISNPNYKVNVYA**^{729}	**1**	**4**	**1**	**2**	**2**	**30**	**1**

The parts of the peptide sequences which were identified in AVA vaccinees (1) are in bold, as are peptides with high binding affinities of <100. Peptide binding capacity was determined by competitive ELISA, with data expressed as relative activity. Means were calculated from at least three independent experiments.

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Response to Comment on "Frequency of Epitope-Specific Naive CD4$^+$ T Cells Correlates with Immunodominance in the Human Memory Repertoire"

We appreciate the comments of Ascough et al. concerning the relevance of immunodominant protective Ag (PA) epitopes that were recently reported by our group (1). We find it equally gratifying that T cell specificities, which were prevalent in the naive repertoire of unvaccinated subjects and the memory repertoire of vaccinated subjects, were also part of the natural immune response towards PA, as such naturally infected subjects were not available for our study. Given the complex regulation of PA and other anthrax toxin genes, differences between the responses of vaccinees and patients would certainly be plausible (2, 3).

We agree that responses to these three peptides are likely to be relevant for a wide section of the population. As summarized in Table I, we have demonstrated using tetramer-guided epitope mapping that these peptides do contain epitopes that are presented and recognized in the context of several common HLA-DR alleles. These observations are in general agreement with the binding affinities observed by Ascough et al. While binding of a given epitope to a particular HLA-DR protein is not a guarantee that it will be recognized in that context, the correlation between these data sets indicates that such binding measurements do have predictive utility.

Table I. PA-specific T cell responses in anthrax vaccine adsorbed vaccinees for several common HLA-DR alleles, identified by tetramer-guided epitope mapping

	HLA-DRB1						
PA Epitope	**01:01**	03:01	**04:01**	**07:01**	10:01	11:01	15:01
$PA_{393-412}$	+	−	+	+	−	−	−
$PA_{505-524}$	+	−	−	+	−	−	−
$PA_{713-732}$	+	+	+	+	+	−	−

Results for HLA-DR that were previously published (1, 4) are indicated in bold. In each column + indicates positive responses observed in multiple subjects and − indicates no response observed.

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