

# Making antigen invisible: a coinhibitory molecule regulates the interaction between T cells and dendritic cells

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**Evaluation of:** Fife BT, Pauken KE, Eagar TN *et al.* Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat. Immunol.* 10(11), 1185–1191 (2009).

Antigen-specific downregulation of T-cell effector function is critical for maintaining self-tolerance but it can promote pathogen persistence in chronic infections; consequently, the restoration of T-cell effector functions is a major goal of therapeutic vaccines against chronic viral infections and malignancies. Recently, a number of T-cell inhibitory receptors, most prominently programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4, have been described that are associated with T-cell exhaustion and tolerance. Blocking these receptors can restore T-cell function and, depending on the model, lead to autoimmune disease or successful viral elimination. Antibodies to PD-1 and cytotoxic T-lymphocyte antigen-4 are currently being tested in clinical trials in several malignant diseases and chronic hepatitis C as they are promising candidates for combination with both prophylactic and therapeutic vaccines. Given the central role of T-cell inhibitory receptors in the regulation of immune responses, understanding their molecular mode of action is of major importance. In the report from Fife and colleagues, two-photon laser scanning microscopy of mouse lymphoid and peripheral tissue has been employed to study the interaction of tolerized PD-1-expressing T cells with antigen-bearing dendritic cells *in vivo*. While tolerized T cells moved freely and did not make prolonged contacts with dendritic cells, addition of an antibody that blocked the interaction between PD-1 and its ligand PD-L1 lowered T-cell motility, enhanced T-cell–dendritic cell contacts and caused autoimmune disease in the nonobese diabetic mouse model of autoimmune diabetes. The authors conclude that PD-1–PD-L1 interactions mediate peripheral tolerance by inhibiting T-cell receptor-induced stop signals.

**KEYWORDS:** anergy • chronic infections • coinhibition • immunity • stop signal • tolerance

The phenomenon of T-cell tolerance is one of the central topics in immunology and is connected to all steps of T-cell development from the thymus to secondary lymphoid organs and peripheral tissues. While tolerance of self and innocuous environmental antigens is critical to avoid autoimmunity and allergic diseases, respectively, inappropriate T-cell unresponsiveness and exhaustion have been implicated in the pathogenesis of chronic infectious diseases and malignancy. In fact, the major aim of

therapeutic vaccination in chronic infections, such as HIV, hepatitis C or hepatitis B, and malignancies, such as melanoma or renal cell carcinoma, is the restoration of T-cell function by overcoming disease-specific T-cell tolerance or exhaustion.

The study by Fife *et al.* investigates the mechanism by which tolerant CD4<sup>+</sup> T cells are inhibited to respond to the tolerogen in a productive fashion [1]. It reports that such cells regulate their early interactions with antigen-presenting

dendritic cells (DCs) via the inhibitory receptor programmed death-1 (PD-1). Although this study arose in the context of autoimmunity, it is of obvious interest to the vaccinologist. Based on exciting work on PD-1 in T-cell exhaustion in chronic infections [2,3], this paper puts forward the idea that the mechanisms underlying T-cell exhaustion and T-cell unresponsiveness for tolerance may be one and the same.

In mice, naive T cells continuously migrate through the lymphatic tissues with a velocity of approximately 10  $\mu\text{m}/\text{min}$  [4]. Only traffic this busy makes it possible for a single DC to interact with approximately 5000 T cells per hour and ensures that the right T cell becomes activated at the right place, and in time to mount an immune response [5]. Upon recognition of antigenic peptide–MHC complexes, T cells reduce their migration velocity drastically and attach to the DC to stabilize the immunological synapse for T-cell receptor (TCR) signaling. Over the following hours, increased calcium levels allow for the induction of gene expression, T-cell activation and proliferation [6].

Fife *et al.* now demonstrate that these early processes do not occur like this when T cells have been previously tolerized. The authors have previously shown that the PD-1–PD-ligand 1 (L1) pathway is involved in the maintenance of peripheral tolerance to self antigens. Activated TCR-transgenic CD4<sup>+</sup> T cells specific for an islet antigen induce diabetes rapidly upon adoptive transfer into animals of the diabetes-prone nonobese diabetic (NOD) mouse strain. However, tolerance can be induced by the injection of antigen-loaded and fixed spleen cells. This work harks back to milestone papers from the laboratory of Ron Schwartz that established a key, one might say paradigmatic, concept in the 1980s. Quill and Schwartz exposed T-cell clones to antigens not by pulsing antigen-presenting cells (APCs) with proteins, but rather with MHC molecules prepared from cell lysates and loaded with the then newly discovered peptide determinants that the clones recognized. The T cells responded by blasting and cytokine production, seen as clear signs of activation. However, when the cells were restimulated with peptide-loaded splenocytes, they were no longer able to proliferate and secrete IL-2. This finding was understood as the direct induction of intrinsic and lasting unresponsiveness by cell-free antigen [7]. When Jenkins and Schwartz found that the fixation of peptide-loaded APCs resulted in similar unresponsiveness, it became clear that APC-derived signals other than peptide–MHC complexes critically contribute to the productive clonal expansion of T cells. In addition, Jenkins and Schwartz showed that a single injection of antigen-loaded and fixed APCs induce unresponsiveness to later immunization with antigen complexed in complete Freund's adjuvant [8]. These seminal observations triggered new avenues of research in understanding the molecular mechanisms of anergy, but also asked what kind of APC-derived signals can trigger them. Now, 20 years later, we are looking at an array of costimulatory and coinhibitory signals that, depending on the assay system, affect or make the difference between tolerance and immunity, and are thus of the highest relevance to the vaccinologist [9]. In addition, several anergy mechanisms have been proposed and confirmed in

mouse models [10–13]. It is such anergy mechanisms, the reviewed paper suggests, that a vaccination against chronic infections has to overcome.

### Summary of methods & results

Having previously established that the injection of antigen-pulsed and fixed splenocytes into NOD mice blocks, and even reverses, the development of spontaneous diabetes in a PD-1-dependent manner, the authors make use of a reductionist model by using TCR transgenic T cells. Although the precise antigen recognized by the BDC2.5 TCR is not known, a peptide mimotope from a synthetic library has been identified and is being used here as a surrogate antigen. A quantity of  $5 \times 10^6$  TCR transgenic T cells activated in culture for 4 days can rapidly induce diabetes in then-healthy NOD animals upon their adoptive transfer. However, the authors demonstrated [14] that the disease is completely blocked by an injection of  $50 \times 10^6$  NOD splenocytes that had been incubated with the mimotope and fixed with the very same chemical used by Jenkins and Schwartz, ethylene carbodiimide [8]. The paper analyzes the differences between autoimmune and tolerized BDC2.5 TCR transgenic T cells.

First the authors ask whether the tolerized T cells resemble anergic ones. It has been reported that anergic T cells do not flux calcium after TCR crosslinking, most likely due to the degradation of proximal TCR signaling components by E3 ubiquitin ligases [15,16]. Indeed, tolerized BDC2.5 cells are unable to mobilize calcium in response to TCR crosslinking. Earlier, the authors had noted that two coinhibitory receptors are being upregulated by BDC2.5 T cells upon activation *in vitro*: PD-1 and cytotoxic T lymphocyte antigen-4 (CTLA-4) [14]. It has now been found that only blocking anti-PD-L1 antibodies, but not controlling or blocking anti-CTLA-4 antibodies, can interfere with tolerance induction. Furthermore, experiments involving a secondary T-cell transfer suggest that PD-1 is also required for the maintenance of anergy in the steady state. These data demonstrate that the PD-1–PD-L1 pathway, but not the one used by CTLA-4, induces and maintains anergy in autoreactive CD4<sup>+</sup> T cells.

To learn more about the behavior of autoimmune and tolerized T cells, activated BDC2.5 cells were labeled with the dye CMTMR and transferred into CD11c-YFP transgenic NOD recipients in which DCs are easily visualized. These animals, in which diabetes rapidly develops, serve as controls for three experimental animals that all received activated BDC2.5 TCR transgenic T cells that were tolerized *in vivo* as described before. These secondary recipients of tolerized cells were then treated with monoclonal antibodies blocking PD-L1, CTLA-4 or a control reagent.

In NOD mice, BDC2.5 T cells recognize pancreatic islet antigens presented by DCs in the draining pancreatic lymph nodes (PLNs), but not in others, for example, in the inguinal lymph nodes (ILNs) [17,18]. The migration velocity of the cells was found to be reduced to approximately 4 and even less than 2  $\mu\text{m}/\text{min}$  12 and 18 h after antigen recognition,

respectively [19]. Statistical analyses of multiphoton time-lapse recordings, which accompany the paper as supplemental items, convincingly demonstrate that:

- Control autoimmune T cells slowed down their motility to approximately 2  $\mu\text{m}/\text{min}$  in antigen-bearing PLNs, but not in ILNs;
- Tolerized T cells moved freely in both PLNs and ILNs with a velocity of approximately 5  $\mu\text{m}/\text{min}$ ;
- Anti-PD-L1 antibodies led to the reduction of cell velocity to less than 2  $\mu\text{m}/\text{min}$  in the PLN;
- Periods of interactions between T cells and DCs were induced that were mostly stable for the duration of the experiments (30 min), while the mean dwell times of tolerized T cells was approximately 9 min;
- The injection of an anti-CTLA-4 antibody made no significant difference to the migratory behavior of tolerized cells.

It has been known for more than 10 years that an early result of antigen recognition by T cells is a stop signal, thus causing the slowed migration and their increased dwell time in contact with APCs. The data indicate that the PD-1–PD-L1 pathway regulates this stop signal between tolerized T cells and DCs *in vivo*; unlike autoimmune control cells, tolerized T cells do not stop in the PLN despite the presence of antigen. Since anti-CTLA-4 antibodies have no effect, the PD1–PD-L1 pathway must operate via at least one, as yet, unknown unique component. It can also be concluded from the data that PD-1 must be engaged at the same time as antigen, as the antibodies have no influence on migratory behavior in the ILN where the DCs do not display islet-derived antigens.

The authors next asked how T-cell motility would be affected in the pancreatic islets of Langerhans, where lymphocyte infiltration is the classical sign of autoimmunity and is taken as a prerequisite for islet destruction. Since direct visualization of pancreatic islets is technically challenging, the authors developed a system whereby islets were transplanted under the kidney capsule (an easily accessible and receptive site) of NOD severe combined immunodeficient animals. For better imaging, the islets were prepared from a NOD donor strain that expresses a green fluorescent protein transgene under the control of the mouse insulin promoter. Tolerized and fluorescently labeled T cells were transferred 1 week later, control and blocking antibodies were applied as before and the T cells' migratory behavior was imaged in explanted islets. While tolerized T cells moved rapidly through the islet, PD-1 blockade led to the lower velocity of approximately 3  $\mu\text{m}/\text{min}$ , unlike the injection of anti-CTLA-4-antibodies

Although no evidence was supplied as to whether the tolerized cells were able to flux calcium and their phenotype was fully reverted by the anti-PD-L1-treatment, functions further downstream were tested, namely phosphorylation of the MAPK Erk and IFN- $\gamma$  production. In tolerized T cells that were isolated from the PLNs 5 h after antibody injection, Erk phosphorylation was indeed increased by anti-PD-L1 treatment. In a similar manner, the T cells infiltrating the pancreas were found to express

IFN- $\gamma$  only upon anti-PD-L1 injection, indicating that previously anergized T cells can become effector cells once the PD-1–PD-L1 pathway is blocked.

### Discussion, expert commentary & five-year view

How the duration of T-cell–DC contacts influences immunity and tolerance induction has been a topic of natural interest to immunologists [6,20,21]. The two main inhibitory receptors and activation markers of T cells, CTLA-4 and PD-1, have both been claimed to regulate the early stop signal triggered by TCR recognition. An earlier paper reported that CTLA-4<sup>+</sup> T cells do not receive the stop signal by TCR triggering, only to be contradicted, however, by a report that T cells from CTLA-4<sup>o/o</sup> animals do not slow down after antigen recognition either [22,23]. The cause of this discrepancy is currently unclear. Fife *et al.* did not find any impact of CTLA-4 antibody injection on migratory T-cell behavior. It may matter that their experiments were exclusively performed in the NOD mouse strain whose T cells express reduced amounts of one of the four CTLA-4 splice variants, which contributes as *Idd5.1* to the diabetes-prone NOD background.

The findings of the reviewed paper are consistent with the fact that CTLA-4- and PD-1-deleted mouse strains exhibit very different autoimmune phenotypes, suggesting that the two molecules operate via different mechanisms. It has also been noted that both receptors carry an immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic tail, while PD-1 contains an additional one, an immunoreceptor–tyrosine-based switch motif. Whether this motif makes the difference in terms of migratory behavior to the T cells, remains to be seen.

For vaccine researchers, the reviewed paper is of interest as PD-1 blockade has been suggested as a novel approach for treating chronic infections. Notwithstanding the undisputed success of prophylactic vaccines against infectious diseases, transferring this approach to the treatment of chronic infections has mainly been a disappointment. Concepts like T-cell tolerance, anergy or exhaustion have been put forward as possible explanations, and animal models, in particular the lymphocytic choriomeningitis virus model in mice, have been exploited to better understand the evolution of T-cell exhaustion and to find means of restoring T-cell function. A milestone observation by Barber *et al.* showed that expression of PD-1 was associated with CD8<sup>+</sup> T-cell exhaustion and blocking the interaction of PD-1 with its receptor PD-L1 could both restore CD8<sup>+</sup> T-cell function and induce viral clearance in mice with prolonged lymphocytic choriomeningitis virus infection [2]. The even more promising approach of therapeutic vaccination combined with PD-1 blockade has already been tested in the same model [24]. Work in chronic human infections such as HIV and hepatitis C has confirmed the role of PD-1 for virus-specific T-cell exhaustion and, at the same time, has fostered hopes for the use of PD-1 antibodies as a treatment of chronic infections [25,26]. A fully human anti-PD-1 antibody (MDX-1106) has been tested in Phase I studies of refractory melanoma, renal cell carcinoma and other solid tumors, and some partial responses have been reported in a population that is very difficult to treat. Even more important

may be the fact that the treatment with anti-PD-1 antibodies did not induce any severe side effects, and only one patient reported arthritic symptoms, which could be a sign of a treatment-induced autoimmune reaction [27]. Based on the work in mouse models, a high risk of autoimmunity must be suspected and the clinical data provide some reassurance, although the number of treated patients was still low ( $n = 21$ ) and most subjects were late-stage tumor patients who must be considered immunocompromised. Early in 2010, results from a Phase I trial with MDX-1106 in patients with chronic hepatitis C are to be expected and, apart from first efficacy data in a chronic viral infection, this will provide important safety data in this otherwise immunocompetent study population.

Meanwhile, however, evidence has accumulated that PD-1 is just one of several inhibitory receptors that are important mediators of T-cell exhaustion [28]. To understand these complex patterns of inhibitory receptors and eventually transfer this knowledge into therapeutic strategies, elucidation of the molecular mode of action of different inhibitory pathways is essential. Here, the Fife *et al.* paper provides important insights into the mechanisms of PD-1-induced T-cell dysfunction. Moreover, it emphasizes the fact that T-cell activation is not just a static on-off phenomenon but rather a complex, multistep sequence of events that has to be studied in the context of a living organism.

Different inhibitory receptors probably interfere at different levels of T-cell activation and function. It may therefore become possible to selectively block distinct pathways as required in a specific disease process.

In conclusion, the therapeutic blockade of T-cell inhibitory pathways, such as PD-1, has opened new avenues for the development of therapeutic vaccines to treat chronic infections and malignancy. Considering the increasingly complex patterns of different inhibitory receptors, elucidation of the molecular correlates of T-cell exhaustion is critical to realize the full potential of this approach. While concerns with regard to the induction of autoimmunity still persist, ongoing clinical trials of anti-PD1 antibodies have so far shown little toxicity, suggesting that even combined blockade of different inhibitory pathways or combination with a specific T-cell vaccine may be feasible.

#### Financial & competing interests disclosure

*The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

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#### Key issues

- Tolerance of self and innocuous environmental antigens is critical to avoid autoimmunity and allergic diseases, respectively. On the other hand, inappropriate T-cell unresponsiveness and exhaustion have been implicated in the pathogenesis of chronic infectious diseases and malignancy. Inhibitory receptors like programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 are critical for the induction and maintenance of T-cell tolerance, and blocking these receptors can restore T-cell responsiveness.
- Using the nonobese diabetic mouse model of autoimmune diabetes, Fife *et al.* investigate the mechanism by which tolerant CD4<sup>+</sup> T cells are inhibited to respond to the tolerogen in a productive fashion. Using two-photon laser scanning microscopy, which allows the study of living cells in the tissue, they found that tolerized T cells moved freely and do not make prolonged contacts with antigen-bearing dendritic cells in lymph nodes. Inhibition of PD-1 lowered T-cell motility, enhanced T cell–dendritic cell contacts and caused autoimmune disease in this mouse model of autoimmune diabetes.
- *In vivo* microscopy of pancreatic tissue, where the autoimmune process takes place, confirmed the influence of PD-1 blockade on T-cell motility at the site of tissue destruction.
- PD-1–PD-ligand 1 interactions mediate peripheral tolerance by inhibiting T-cell receptor-induced stop signals.

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