

# Immunogenic and tolerogenic effects of the chimeric IL-2-diphtheria toxin cytotoxic agent Ontak® on CD25<sup>+</sup> cells

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**Abbreviations:** DC, dendritic cell; T<sub>conv</sub>, conventional T cell; T<sub>reg</sub>, regulatory T cell; IL-2, interleukin-2; IL-2R, IL-2 receptor; DT, diphtheria toxin

Ontak®, a conjugate between IL-2 and a diphtheria toxin fragment, was recently investigated in cancer clinical trials aiming to kill CD25<sup>+</sup> regulatory T cells (T<sub>regs</sub>). We found that the activity of Ontak® was more complex on T<sub>regs</sub> and conventional T cells (T<sub>conv</sub>) than anticipated, including a novel strong influence on dendritic cells (DCs).

Pharmacological intervention strategies aiming to modulate immune responses include toxins conjugated to ligands that recognize cognate receptors on target cells. Ligand-receptor binding purportedly initiates internalization of the toxin-ligand-receptor complex, thereby affecting target cell killing. Such simplistic approaches, however, postulate rudimentary biological functions that may, in actuality, be far more complex processes in vivo.

Immunization with vaccines against tumor antigens or infusion of cancer-specific T cells is expected to ablate residual malignant cells and reduce metastatic burden. However, activation of the adaptive immune system naturally elicits the counter-activity of endogenous tolerance mechanisms. These include the immunosuppressive activity mediated by T<sub>regs</sub> functioning to maintain self-tolerance. Tregs also limit and ultimately terminate immune responses against foreign antigens. Since tumors are composed of autologous cells typically exhibiting moderate immunological phenotypic alterations relative to the originating tissue cell type, T<sub>reg</sub> infiltration and immunosuppressive activity is frequently observed to

occur at tumor sites. Therefore, either the direct pharmacological depletion of T<sub>regs</sub>, or alternatively, the application of agents designed to influence their differentiation state or immune-inhibitory properties, could enhance native anticancer immune responses. One strategy to deplete T<sub>regs</sub> is to target them with Ontak®, a fusion molecule of the truncated coding fragment A and the membrane-associated domain B of diphtheria toxin genetically linked to human interleukin-2 (IL-2).<sup>2</sup>

Cellular toxicity of Ontak® requires the IL-2 receptor (IL-2R)-mediated uptake of the chimeric toxin into acidic intracellular vesicles and subsequent transfer into the cytoplasm where it binds to the eukaryotic translation elongation factor 2 (EEF2) inhibiting protein synthesis precipitating apoptotic cell death.<sup>3</sup> Ontak®-susceptible cell types express the  $\alpha$  chain for the IL-2R, more commonly known as CD25<sup>+</sup>. CD25 is present on lymphoid cancer cells, mature dendritic cells (DCs), activated and antigen-specific conventional T cells (T<sub>conv</sub>), and T<sub>regs</sub>, all of which also constitutively express both the IL-2R  $\beta$  (CD122) and common  $\gamma$  chains (CD132) to form the high affinity IL-2R $\alpha\beta\gamma$  complex.

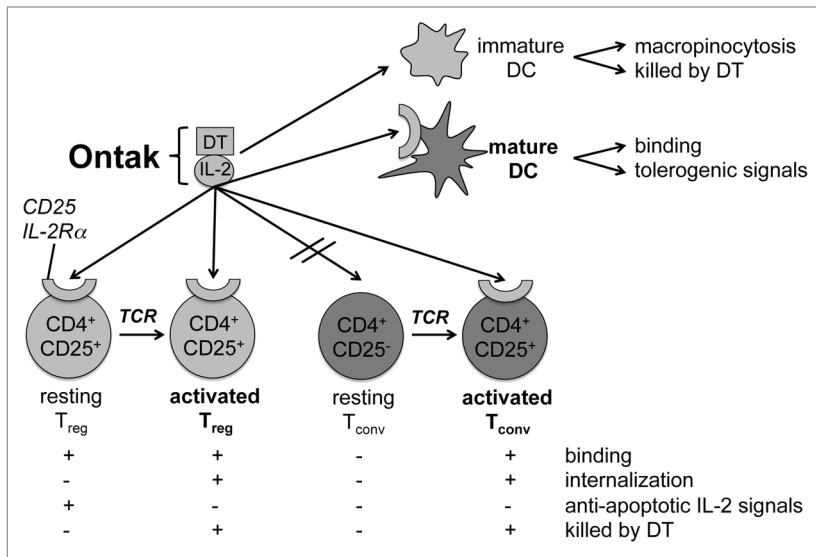
The hypothesis that CD25<sup>+</sup> leukemia and lymphoma cells could be killed by cytotoxic therapy with Ontak® was tested in the first clinical trials using this drug. Since then successful application of Ontak® against undesirable CD25<sup>+</sup> T<sub>conv</sub> responses has been reported in mouse models in vivo<sup>4</sup> and in studies assaying human cells in vitro.<sup>5</sup> These studies implied that Ontak® could potentially dampen overshooting immunologic responses or treat autoimmune-related pathologies (such as psoriasis) leading to clinical trials with good therapeutic outcomes.<sup>6</sup>

More recently, cytolytic effects of Ontak® on immunosuppressive CD25<sup>+</sup> T<sub>regs</sub> have been reported. Injection of Ontak® into mice has been observed to deplete T<sub>regs</sub> and enhance T-cell immunity.<sup>7</sup> However, in the non-obese diabetic (NOD) mouse model of human type-1 diabetes, we reported that Ontak® either promoted disease or had no effect, depending on the time point of application.<sup>8</sup> Several clinical studies (cited in ref. 1) reported the successful depletion of T<sub>regs</sub> by Ontak® in treated cancer patients, although others have failed to detect Ontak®-mediated T<sub>reg</sub> depletion in melanoma patients.<sup>9</sup> The

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**Figure 1.** Binding of Ontak<sup>®</sup> to DC, T<sub>conv</sub> and T<sub>reg</sub> results in diverse effects. The IL-2 conjugate with diphtheria toxin (DT; i.e., Ontak<sup>®</sup>) binds to the high affinity IL-2R dictated by the  $\alpha$  chain (CD25) component that associates with the constitutively expressed  $\beta$  and  $\gamma$  chains. Immature DCs do not express CD25 but internalize Ontak<sup>®</sup>, most likely via macropinocytosis, leading to their toxin-mediated killing. Mature CD25<sup>+</sup> dendritic cells (DCs) bind Ontak<sup>®</sup> resulting in the stimulation of tolerogenic-signaling pathways. Resting CD25<sup>+</sup> regulatory T cells (T<sub>reg</sub>) bind Ontak<sup>®</sup> but do not internalize it. Therefore, the IL-2 component of Ontak<sup>®</sup> rather transmits anti-apoptotic signals through the IL-2R. Resting CD25<sup>-</sup> conventional T cells (T<sub>conv</sub>) remain unaffected due to a lack of Ontak<sup>®</sup> binding. Both T cell receptor (TCR)-activated T<sub>conv</sub> and T<sub>reg</sub> bind and internalize Ontak<sup>®</sup> and are, therefore, subsequently killed by the toxin.

reasons underlying these incongruous outcomes are obscure.

Our own clinical data obtained from Ontak<sup>®</sup>-treated melanoma patients shortly before undergoing tumor-specific DC vaccinations indicated that Ontak<sup>®</sup> prevented the development of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses against the vaccine.<sup>1</sup> This prompted us to investigate the effects of Ontak<sup>®</sup> on immature and mature DCs, as well as resting and activated T<sub>reg</sub> and T<sub>conv</sub>, in more detail *in vitro*.

One outstanding question was whether Ontak<sup>®</sup> could selectively target CD25<sup>+</sup> T<sub>reg</sub> or CD25<sup>+</sup> T<sub>conv</sub> in different diseases, and if so, how, considering that both cell types are simultaneously present in lymphoid and effector tissues during autoimmunity and tumor-immunity. In our patients we failed to detect reliable depletion of Tregs after Ontak<sup>®</sup> application. Thus, we investigated the susceptibility of resting and activated T<sub>reg</sub> and T<sub>conv</sub> to Ontak<sup>®</sup> *in vitro*.<sup>1</sup> Our results indicate that the cytotoxicity of Ontak<sup>®</sup> correlates with the activation state of both T-cell types (Fig. 1). At a resting state, high doses of Ontak<sup>®</sup> are required to induce apoptosis

in both cell types whereas low doses are anti-apoptotic. In contrast, freshly activated T<sub>reg</sub> and T<sub>conv</sub> are both readily killed by Ontak<sup>®</sup>.

Additionally, we found a totally novel effect of Ontak<sup>®</sup> on DCs. Surprisingly, both immature and mature DCs were affected, thereby influencing both the tolerogenic and immunogenic stages of this antigen-presenting cell concurrently. Immature, CD25<sup>-</sup> DCs bound and ingested Ontak<sup>®</sup>, most likely by macropinocytosis, and then underwent massive apoptosis in a dose-dependent manner. In contrast, mature CD25<sup>+</sup> DC were killed only at high doses of Ontak<sup>®</sup>, whereas low dosages downregulated essential costimulatory molecules such as CD83, CD70, and CD25 (Fig. 1).<sup>1</sup> The latter finding is fatal, since Ontak<sup>®</sup> converts mature DCs that would normally initiate anticancer T-cell immune responses into an immature DC-phenotype capable of fostering tolerance against the presented tumor antigens.

Our combined *in vitro* and *in vivo* data demonstrate that Ontak<sup>®</sup> cannot selectively deplete or inactivate tolerogenic cells

such as T<sub>reg</sub> and immature DCs while successfully eliminating immunogenic cells such as activated T<sub>conv</sub> and mature DC. The discrepancies between human reports in regards to the selective depletion of T<sub>reg</sub> may be due to differences in the activation states of T<sub>reg</sub> present in various diseases, disease stages, or under the influence of parallel pharmacological treatments. In any case, more rigorous analyses of each patient's unique immune system should be performed before Ontak<sup>®</sup> treatment. For further clinical applications of Ontak<sup>®</sup> beyond its approved use in T cell lymphoma, these multifaceted effects have to be considered encompassing T cell and DC subsets and their corresponding activation stages. The outcomes of Ontak<sup>®</sup> immunotherapy in the treatment of different human diseases cannot be easily predicted due to its complex biological activity.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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