all these organisms was almost certainly a nonphotosynthetic anaerobe.

The timing of these evolutionary events in relation to the great oxidation event remains uncertain. Substantial geological evidence suggests that the ability of Cyanobacteria to produce O_2 may have predated the great oxidation event by as much as 600 million years and that the accumulation of O_2 in the atmosphere was delayed because reduced species such as Fe^{2+} in the ocean first had to be oxidized (*10*). In contrast, Shih *et al.* have used molecular clocks to date the divergence of the photosynthetic from the nonphotosynthetic Cyanobacteria at 2.5 billion to 2.6 billion years ago; this date is much closer to the great oxidation event (*11*). This is unlikely to be the final word on this issue, however, because accurate molecular clock–dating of bacteria is notoriously difficult because of a lack of well-defined constraints from the fossil record (*12*).

On the basis of Soo *et al.*'s findings, it is probable that the ancestors of the photosynthetic Cyanobacteria were not themselves phototrophic (capable of obtaining energy from sunlight). This line of bacteria therefore either had to develop this metabolic capability de novo or import it via horizontal gene transfer. The latter is almost certainly the case, given the clear evolutionary and mechanistic similarities in all organisms capable of chlorophyll-based phototrophy (*13*). The origin of oxygenic photosynthesis may thus be described as resulting from the horizontal gene transfer of information needed for this metabolic process to a previously nonphotosynthetic line of organisms (see the figure). This group became the photosynthetic Cyanobacteria, which went on to develop the ability to oxidize water and changed the world. ■

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IMMUNOTHERAPY

Costimulation, a surprising connection for immunotherapy

T cell cosignaling molecules may determine sensitivity to immunotherapy

By **Derek L. Clouthier**[1] *and* **Pamela S. Ohashi**[1,2]

Checkpoint blockade is a type of immunotherapy that has shown unprecedented success in treating many cancers (*1*), particularly blockade of the T cell checkpoint protein called programmed cell death–1 (PD-1). This has created a unique situation in which clinical studies have outpaced efforts at the bench. As such, reliable predictive biomarkers have not yet been identified that define who will benefit from this method of treatment, and there is only a partial understanding of the mechanisms of sensitivity or resistance to immunotherapy. On pages 1428 and 1423 of this issue, Hui *et al.* (*2*) and Kamphorst *et al.* (*3*), respectively, elucidate important mechanisms of checkpoint blockade by demonstrating that PD-1 exerts its primary effect of dampening T cell activation by regulating a T cell receptor costimulatory molecule called cluster of differentiation 28 (CD28).

Naïve T cells are activated by antigen-presenting cells (APCs) in lymphoid organs, such as lymph nodes, before migrating to the tumor or site of infection. Immune checkpoint inhibitors are thought to act at different stages of the T cell's journey from activation in lymphoid organs to the tumor (see the figure). Although full T cell activation requires cognate antigen stimulation through the T cell receptor (TCR; signal 1) and costimulation (signal 2) from APCs, understanding of T cell activation has evolved far beyond the "two-signal" model initially posited in the 1970s (*4*). Dozens of stimulatory and inhibitory T cell cosignaling molecules fine-tune T cell responses (*5*), and most are being heavily investigated as immunotherapeutic targets. The first two immune checkpoints to be successfully blocked in the clinic are cytotoxic T lymphocyte–associated protein-4 (CTLA-4) and PD-1. CTLA-4 competes with CD28 for the same ligands on APCs [B7.1 (CD80) and B7.2 (CD86)], thereby regulating T cell activation by limiting costimulation. Expression of CTLA-4 on activated T cells (effector T cells) increases after TCR stimulation and is thought to control T cell activation that is elicited by APCs. PD-1 is also expressed after TCR stimulation, but is typically thought to regulate T cell responses at the tumor (or site of infection). One rationale for this scenario is that expression of PD-L1, a ligand for PD-1, is inducible on all cells by inflammatory signals, whereas the ligands for CTLA-4, B7.1 and B7.2, have largely restricted expression to professional APCs. PD-1 has another ligand, PD-L2, which is also restricted to APCs (*6*).

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> ***"Understanding...T cell cosignaling molecules... will be essential for safe and effective combination immunotherapies."***

When exposed to persistent antigen, such as in the setting of cancer or chronic infection, cytotoxic $CD8^+$ T cells become functionally "exhausted," such that they progressively lose proliferative capacity, cytokine production, and cytolytic activity. It is currently thought that one of the main effects of PD-1 blockade is to reverse T cell exhaustion (*7*).

Kamphorst *et al.* demonstrate the necessity of CD28 signaling (upon interaction with B7.1 or B7.2) for restoring T cell responses during blockade of PD-1 (treatment with anti–PD-1 antibody) in a mouse model of viral infection. Clinical samples from non–small cell lung cancer patients undergoing PD-1 blockade also revealed that $CD8^+$ T cells expressing CD28 preferentially responded to PD-1 blockade. Hui *et al.* performed elegant biochemical studies demonstrating that PD-1, but not CTLA-4, recruits the Src homology 2 domain–containing phosphatase (Shp2) to dephosphorylate PD-1 itself, as well as CD28. This biochemical modification terminates CD28 signaling. When T cells (transfected to express CD28 and PD-1) were exposed to a lipid bilayer bearing several proteins—major histocompatibility complex class I (MHC I),

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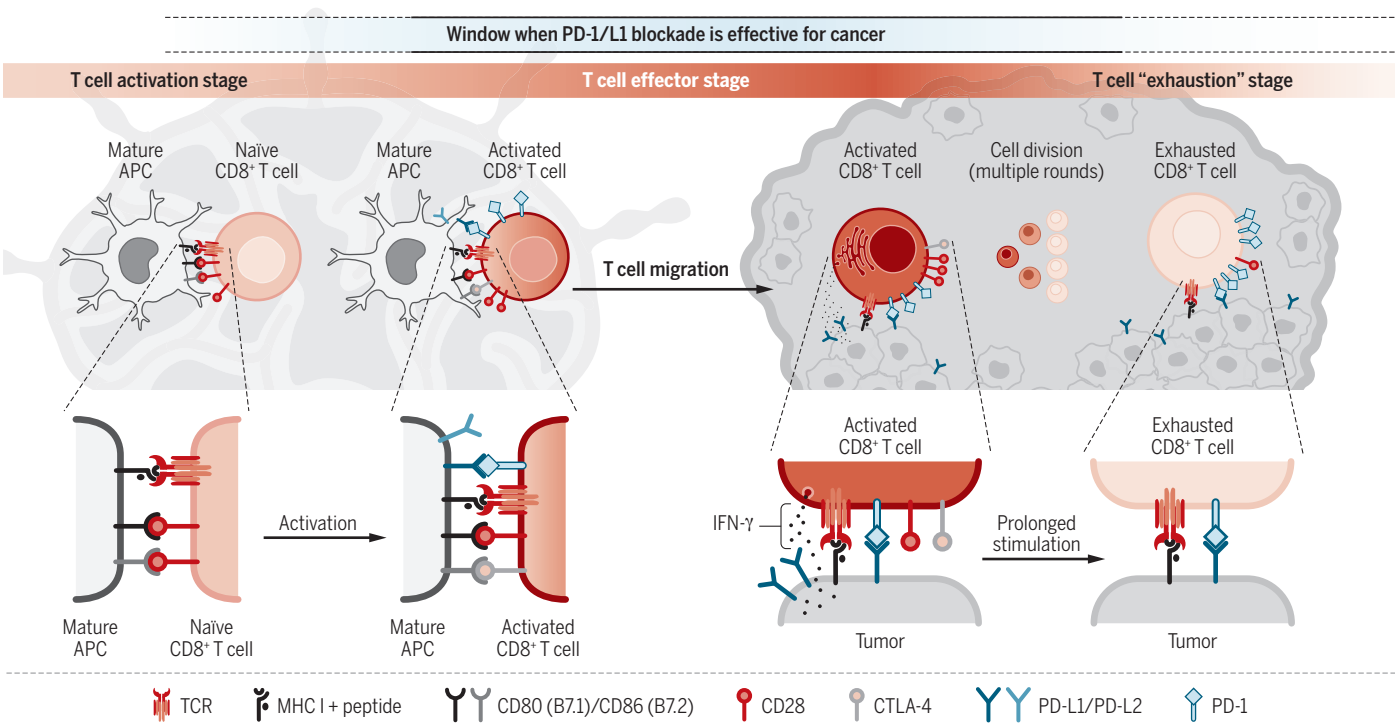
PD-1 blockade, when and where?

Blockade of PD-1 at the time of T cell activation increases the number and functionality of T cells. It is unclear whether this can also happen in the tumor. "Exhausted" T cells highly express PD-1 but are not responsive to PD-1 blockade, perhaps due in part to the loss of CD28. IFN-γ, interferon-γ



intercellular adhesion molecule 1, B7.1, and PD-L1—the CD28 and PD-1 proteins clustered centripetally around the TCR within 30 seconds, leading to the dephosphorylation of CD28 by PD-1. The TCR and its signaling components were the assumed targets of PD-1 and SHP-2, but Hui *et al.* show that CD28 is a more sensitive target, followed by lymphocyte-specific protein tyrosine kinase (Lck), the enzyme that phosphorylates the TCR signaling complex, CD28, and PD-1. These findings are surprising because PD-1 blockade is thought to act on "exhausted" T cells (those with progressive loss of function) rather than during the activation and effector phases of the T cell response. More importantly, CD28 was not a suspected target of PD-1.

The findings of Hui *et al.* and Kamphorst *et al.* are supported by earlier studies and emerging lines of evidence demonstrating that PD-1 blockade targets T cells that are not yet exhausted. In mice, self-renewing short-term memory $CD8^+$ T cells that express an intermediate amount of PD-1 ($PD\text{-}1^{int}$) also express higher amounts of costimulatory molecules (including CD28) and selectively expand in response to PD-1 blockade (*8*). More severely exhausted T cells expressing a high amount of PD-1 ($PD\text{-}1^{hi}$) coexpress other negative regulators and lose expression of costimulatory molecules (including CD28) and are nonresponsive to PD-1 blockade. CD28 is also lost by human $CD8^+$ T cells following long-term antigen stimulation (*9*). As such, it is important to consider a role for PD-1 blockade in the early stages of the T cell response. PD-1 limits the initial proliferative burst of T cells at the time of activation by antigen, and PD-1 blockade can tip the balance from tolerance induction to effector differentiation (*10*). Early work suggested that PD-1 restrains T cell activation in a CD28-dependent manner (*11*). Taken together, these studies support a model in which PD-1 blockade acts during T cell activation and immune surveillance as opposed to the traditional notion of "reversing exhaustion." The findings of Hui *et al.* and Kamphorst *et al.* provide further rationale to combine PD-1 blockade therapy with treatments aimed at generating de novo immune responses, such as tumor vaccines or oncolytic viruses.

PD-1, CD28, and CTLA-4 are not redundant signaling pathways. Although the studies of Hui *et al.* and Kamphorst *et al.* show that CD28 is necessary for the effect of PD-1, the reverse is not necessarily true. CTLA-4 and PD-1 induce unique cellular effects, and mice engineered to lack either receptor clearly have different phenotypes (*6*). Moreover, combination CTLA-4 blockade and PD-1 blockade clearly show synergistic effects in melanoma and non–small cell lung cancer (*12*–*14*). It is possible that the synergistic effects are due to the action of anti–CTLA-4 antibodies on regulatory T cells that express a high amount of CTLA-4. In this case, regulatory T cells may be prevented from exerting immunosuppressive functions, including competing with CD28 for binding to its cognate ligands (*15*). Additional hints at cross-talk between PD-1 and CD28 come from studies that show PD-1 blockade is most effective in patients with tumor-infiltrating immune cells that express PD-L1 (*1*). These immune cells would also express ligands for CD28, which are otherwise absent in most tumors.

Understanding the kinetics of expression of T cell cosignaling molecules and their ligands and how their signals interact will be essential for safe and effective combination immunotherapies. The findings of Hui *et al.* and Kamphorst *et al.* may also provide guidance on developing predictive biomarkers for immuno-oncology agents in the clinic. ■

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