TPO signaling: when the tyrosines go marching in(side)

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**TPO signaling: when the tyrosines go marching in(side)**

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In this issue of *Blood*, Hitchcock and colleagues provide novel insights into the molecular pathways regulating TPO-mediated c-Mpl trafficking, highlighting the essential role of AP2 in receptor internalization and lysosomal degradation as new pieces in the TPO/c-Mpl signaling puzzle.

Thrombopoietin (TPO) and its receptor c-Mpl are the primary regulators of megakaryocytosis and play a critical role in hematopoietic stem cell biology. Upon TPO binding, c-Mpl facilitates tyrosine (Y) phosphorylation of cytoplasmic signaling proteins and activation of several pathways that control cellular proliferation, megakaryocyte development, and survival. Turning off the TPO signal is critical to prevent uncontrolled proliferation; however, the molecular processes governing this process are still not fully elucidated. Suppressors of cytokine signaling (SOCS), phosphatases, and other proteins, such as focal adhesion kinase and members of the Src family kinases (SFKs), have recently emerged as negative modulators of TPO signaling.1,2

Although it is still a matter of debate, soon after the discovery of TPO it was suggested that internalization of TPO/c-Mpl complexes by platelets and megakaryocytes is a primary means of regulating plasma TPO levels. A phosphotyrosine (Y112) and 2 dileucine motifs (L54L55 and I57L58) within the cytoplasmic domain of the receptor have been identified to promote TPO-mediated c-Mpl internalization and proliferative signaling. Furthermore, c-Mpl recycling from intracellular pools has also been demonstrated.3

Clathrin-mediated endocytosis constitutes the major route for selective receptor internalization in higher eukaryotes. Clathrin molecules are recruited to the cell surface where they interact with transmembrane proteins via adaptor proteins (AP) such as AP2 to form clathrin-coated pits. YXXφ and [DE][K][I][L] motifs in the cytoplasmic tails of transmembrane proteins have been identified as the binding sites for AP2.4

Hitchcock et al, in a series of elegant experiments, have demonstrated that AP2 has a key role in TPO-stimulated clathrin-mediated c-Mpl internalization in both Jak2 and SFKs activation-dependent pathways. Bearing in mind that c-Mpl contains 2 YXXφ motifs in its cytoplasmic domain at Tyr8 (Y8RRL) and Tyr78 (Y78RRL) and using BaF3-Mpl–expressing cells with point mutations at Y8 or Y78, the authors have identified Y78RRL as the binding motif for AP2 in the c-Mpl receptor. Interestingly, they also observed that cells expressing the Y78F mutation exhibited increased proliferation and prolonged activation of Jak2, STAT5, AKT, and ERK1/2 in response to TPO. The observation that knocking down AP2 did not have the same effects on signaling as the mutation led the authors to suggest that Y78 may be responsible not only for the internalization but also for the activation of other signaling pathways that restrain the TPO signal.

Although the clearance of c-Mpl was recently attributed only to the proteasome,5 this study unveiled that lysosomal degradation is another control mechanism of c-Mpl expression and also identifies Y8RRL as the lysosomal targeting motif. Accordingly, Y8F c-Mpl cells showed an increase in c-Mpl recycling to the cell surface (see figure).

This paper is the first report that describes the YXXφ motif for receptor internalization and lysosome targeting in the hematopoietic growth factor receptor family and raises many new questions that await an answer. What
mechanisms control the balance between c-Mpl degradation/recycling? What contribution does c-Mpl internalization make to the TPO proliferative signal? What would be the biological impact of the c-Mpl Y75 and Y8 mutations in primary cells? To what extent are Y mutations linked to the pathogenesis of myeloproliferative diseases? Investigating these questions should help us to understand the interplay between the actors controlling the TPO/c-Mpl scenario.

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Test-driving CARs

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Redirecting T-cell specificity through the introduction of a chimeric antigen receptor (CAR) is emerging as a clinically feasible approach for adoptive immunotherapy. In this issue of Blood, Till and colleagues now demonstrate that autologous T cells genetically modified to express a CD20-specific CAR can be safely infused in patients with B-lineage lymphomas.

Approximately 15 years after Zelig Eshhar demonstrated how to redirect the specificity of T cells, investigators are reporting their early clinical experiences with infusing T cells genetically modified to express chimeric antigen receptors (CARs). By combining T-cell therapy with gene therapy in compliance with current good manufacturing practice (cGMP) for phase 1 and 2 trials, investigators have bypassed tolerance to enable clinical-grade T cells to recognize desired cellsurface antigens independent of MHC. T cells are rendered tumor-specific through introduction of a CAR, which is typically composed of the scFv from a monoclonal antibody (mAb) that forms part of the CAR ectodomain and which, upon binding antigen, activates T cells by phosphorylation of conserved immunglobulin tyrosine activation motifs within a chimeric CD3-ζ or FcεRI endodomain. To limit potential deleterious off-target effects, the first human trials for hematopoietic malignancies have infused genetically modified CAR+ T cells that target lineage-restricted antigens, such as CD19 and CD20 expressed on malignant (and healthy) B cells, and generally have used first-generation CARs (activating T cells solely through CD3-ζ). Building on clinical experiences of therapeutic mAbs targeting CD20, Till and colleagues demonstrate that an intrapatient dose-escalation study infusing autologous clinical-grade T cells expressing a CD20-specific CAR with or without low-dose IL-2 can be undertaken in patients with non-Hodgkin lymphomas. In the current financial and regulatory climate, publishing the results of a gene-therapy trial is a singular accomplishment. However, like many initial human experiences, the results raise more questions than they answer.

Sustaining the survival of adoptively transferred CAR+ T cells is one of the major impediments to achieving significant therapeutic responses. One way to enhance persistence is to infuse a heterogeneous population of T cells so that subpopulations can participate in lymphopenia-induced proliferation. This has been championed by Rosenberg and colleagues at the National Institutes of Health who demonstrated that clinical responses can be achieved when melanoma-specific T cells are infused after lymphodepleting chemotherapy and when bulk populations of T cells are given rather than T-cell clones. Thus, most infusions of CD20-specific T cells were given after chemotherapy and the trial was altered to infuse populations of genetically modified T cells rather than clones. Cumulatively, up to 4.4 × 10^9/m^2 cells were infused within 10 days, and while a limited number of infused T cells could be detected in the peripheral blood of some patients for up to 3 months, the question remains as to why the infused T cells did not persist longer and at increased levels leading to loss of normal CD20+ B cells. Perhaps it was due to the cells entering replicative senescence after nonviral gene transfer and ex vivo expansion to clinically meaningful numbers. Perhaps it was due to insufficient T-cell help, despite the use of low-dose IL-2. Perhaps it was due to the level of CAR expression or competency of CAR-dependent signaling, which might have been insufficient to sustain a proliferative T-cell signal. Perhaps it was due to a lack of central-memory T cells in the inoculum that were capable of long-term in vivo persistence. Or perhaps it was due to incomplete lymphodepletion resulting from the choice of pre-infusion chemotherapies used.

The trial described by Till et al drives home the observation that multiple infusions of autologous CAR+ T cells targeting a B-lineage antigen are both safe and feasible. This clinical experience can now serve as a platform for future endeavors to answer questions concerning the improvement of persistence and the resolving of issues regarding homing to tumor deposits, thereby improving the therapeutic potential of their CAR+ T cells.

The clinical data in this issue of Blood are among the first reports on the potential of T cells manufactured under cGMP that have been genetically modified to redirect specificity. With this publication, the authors have advanced the promising technology of CARs, which combine the specificity of mAbs with the replicative and homing potentials of T cells.

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