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Pillars of Immunology

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Opening the Black Box of Immunosuppression

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In the last quarter of the twentieth century, immunologists began to shine light into one of the most mysterious black boxes: how does the extracellular environment impact gene expression in the nucleus? At the same time, the NF-κB and STAT pathways were being identified, ultimately leading to an understanding of cytokine signaling; the pathways between the TCR and the nucleus were similarly being defined. Ag receptor engagement stimulated activation of NF-κB, AP-1, and a novel factor identified in EMSAs as the NF-AT (1). This was found to be a multiprotein complex, with the NFAT proteins (as the cloned genes became known) identified as a preformed subunit in the cytoplasm of resting T cells (2).

What we now understand is that T cell activation through the TCR activates intracellular calcium flux, which in turn activates calcineurin (3). Calcineurin dephosphorylates NFAT proteins in the cytoplasm, allowing their transit to the nucleus (2), DNA binding, and transcriptional activation of numerous genes, including those encoding cytokines. Calcineurin inhibitors, a class of immunosuppressant small molecules that includes cyclosporine A (CsA) and FK506, bind to immunophilins (4). The immunophilin/drug complexes bind calcineurin and inhibit phosphatase activity, preventing NFAT nuclear localization and gene activation (4).

This sequence of events is learned by budding immunologists in undergraduate immunology courses or, perhaps for those who stay immunodeficient slightly longer, in the first year of graduate school. But almost 30 years ago, when the *Pillars of Immunology* article by Emmel et al. (5) was published, almost none of that had been defined. Through a series of experiments that are now conceptually simple but at the time were elegantly insightful, Emmel and colleagues in the Crabtree laboratory made a significant impact in answering how calcineurin inhibitors blocked T cell activation.

Calcineurin inhibitors, particularly CsA, had been used as potent immunosuppressants and were being used in the clinic to revolutionize organ transplantation, because they could minimize rejection. A history of calcineurin inhibitor development and usage was recently published in The Journal of Immunology (4). From many studies, it was known that CsA inhibited T cell activation, proliferation, and, specifically, the production of IL-2. Yet how it achieved this profound immunosuppression was still not entirely clear.

To begin to address this question, Emmel et al. (5) asked which transcription factor was actually affected by CsA. Using reporter assays and response elements from the *IL2* gene with mutated binding sites for specific factors, the authors tested for differences in inhibition of the response to CsA. Several initial candidates were identified, including AP-1, NF-AT, and NF-κB. The authors then compared the sensitivity of concatemers for specific factors with the intact *IL2* enhancer and observed that CsA blocked reporter activity from the *IL2* enhancer and the NFAT concatemer but not an AP-1–responsive element. An NF-κB reporter was also inhibited by CsA, but NF-κB activity was not as sensitive as NFAT-dependent gene activation.

These results suggested NFAT was a prime candidate. To follow up on this observation, the authors then tested whether binding of any of these factors to DNA was affected by CsA using EMSA. Jurkat cells were stimulated in the presence or absence of CsA, and nuclear extracts were used for the EMSA. They observed that, although NF-κB and AP-1 binding were not affected by CsA, NF-AT binding was decreased. This further confirmed that NF-AT was a potential CsA target.

Finally, the authors tested these findings in vivo. They generated transgenic mice that had an NFAT binding concatemer (4×) upstream of the *IL2* promoter, both upstream of the SV40 T Ag. They showed in this article and in a parallel report that without the NFAT binding concatemer, the *IL2* promoter did not promote transcription of SV40 T Ag when splenic lymphocytes were stimulated with PMA and ionomycin or anti-CD3 (5, 6). However, when the concatemer was linked to the *IL2* promoter, T cell activator–induced transcription of SV40 T Ag was observed. This provided the opportunity to test CsA activity on the reporter transgene. Indeed, when the NFAT concatemer transgenic cells were stimulated in the presence of CsA, the SV40 T Ag gene was not transcribed.

The finding that AP-1 site mutations of the *IL2* enhancer affected CsA responsiveness, although the AP-1 concatamer did not, foreshadowed one of the subsequent important findings in NFAT biology. Early studies suggested that there were both preformed and induced components of the NF-AT complex (7). It was subsequently found that AP-1 was the induced component and that NFAT and AP-1 had significant interactions on DNA elements (2).

How the immunophilins dictated NF-AT activity was still not clear at the time of this Pillar report. There was considerable discussion at the time (and in this article) about the potential for the peptidyl-prolyl activity of the immunophilins

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Abbreviation used in this article: CsA, cyclosporine A.

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to impact NF-AT or even calcineurin activity. But subsequent studies and reviews argued against this mechanism (8). Ultimately, it was the complex of the calcineurin inhibitor and the immunophilin that was shown to inhibit calcineurin activity (9).

The complexity of the NFAT family members was also realized later (10). The five family members that are currently known have considerable functional overlap and, although mice deficient in a single NFAT family member had phenotypes, those deficient in two or more family members demonstrated dramatically compromised NFAT activity, resulting in immune phenotypes that were significantly altered (11–16). Gene-deficient mice also highlighted additional cell types in which NFAT signaling is important, including heart development, chondrogenesis, and neuronal growth (17–20).

CsA and related immunosuppressants have remained important components in the treatment of various immune-related disorders, including transplant rejection and autoimmune disease. As with many drugs, clinical use preceded a detailed understanding of how the drug worked on a molecular level. Yet the "black box" between signal and mediator began to grow a little less mysterious with this Pillar and cotemporaneous papers. These insights not only allowed researchers to understand CsA targets but also advance use of the drug as a tool to further define NFAT activation and function.

Disclosures

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