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Measurement of multiple soluble cytokines and the activated cells secreting them in whole blood

Immune responses are complex events requiring the synchronized participation of cells and soluble factors. Secreted cytokines play a major role in the coordination of the immune response by inducing the activation or inhibition of specific cell populations through cytokine receptors. Monitoring this extremely intricate system is not an easy task. Numerous methods are available to identify cells secreting cytokines based on messenger RNA (mRNA) measurements in bulk cell preparations, or of single cells using *in situ* techniques. However, analysis of the secreted protein, which could be performed by Western or ELISA methods, is preferred. More recent assays, such as the ELISPOT and those based on flow cytometric technologies, allow not only the measurement of the secreted cytokine but also the identification of the cells that produce them. The disadvantage of these assays is that they are complex, and require significant sample manipulation, which may alter the physiological state of the system being analyzed.

In this issue, **Rodriguez-Caballero *et al***[1] (p. 1387) report a new approach to simultaneously identify specific cytokine-producing cells and quantify multiple cytokines secreted by these cells using a relatively simple flow cytometric-based immunoassay applied to unfractionated blood samples. To measure simultaneously cell activation and cytokine secretion, tumor necrosis factor α (TNFα), which is used as a universal marker of cytokine-secreting cells, is stabilized at the cell surface by a specific inhibitor of the TNFα-converting enzyme. This allows the determination of the phenotype of TNFα-secreting cells while at the same time, and in the same tube, multiple secreted cytokines are quantified using commercially available fluorescent beads coupled with cytokine-specific monoclonal antibodies. The distinct light scatter properties of beads and cells allow their easy discrimination by flow cytometry. The authors not only show that this method could be used for the evaluation of responses by blood cells against generic stimuli such as lipopolysaccharide and PMA/ionomycin, but also to distinguish cytomegalovirus (CMV)-seropositive from CMV-seronegative individuals. In addition to quantitating different soluble cytokines, the authors identified the monocytes and T-cell subsets responsible for CMV-induced cytokine secretion, a finding that provides an opportunity to investigate the phenotypic and functional characteristics of CMV-specific T cells.

The simple but informative single-tube, flow cytometric measurement of multiple cytokines and the cells responsible for their production described in this article should be an excellent tool in the direct assessment of immune responses in whole blood samples under a variety of physiological and pathological conditions.

References

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Exact RNA analysis of splice site fidelity: a blistering problem

Gene transcription generates a pre-mRNA. Spliceosomes then remove introns and ligate exons, leading to formation of mature mRNA. This process critically depends on accurate recognition of correct splice sites by the spliceosomes. Splice sites are highly conserved sequences at the exon–intron border; variations in the consensus nucleotide sequences can generate 'weaker' or 'stronger' splice sites. Mutations can inactivate a splice site, leading to spliceosome targeting of sequences resembling consensus sequences in legitimate introns or exons. Such 'cryptic splicing' usually leads to loss of coding sequences, inclusion of noncoding sequences, frameshifts, and premature termination codons. The mRNA surveillance mechanism, nonsense-mediated mRNA decay (NMD), selectively targets defective mRNAs with premature termination codons for rapid degradation. On rare occasions, a mutation elsewhere in a gene can generate a new splice site.

Junctional epidermolysis bullosa (JEB) is a blistering skin disorder characterized by separation within the lamina rara of the dermo-epidermal junction. It results from a reduction or total lack of laminin-5, a scaffolding protein that plays a critical role in adhesion of basal epidermal cells to the collagen matrix of the basement membrane. Laminin-5 is a heterotrimer of three polypeptide chains: α3, β3, and γ2, encoded by the genes LAMA3, LAMB3, and LAMC2, respectively. The most severe form of JEB results from complete loss of the laminin-5 protein, and is usually lethal within the first 2 years of life. Mutations leading to premature termination codons

on both alleles of the LAMB3 gene, and hence NMD deletion of the respective mRNAs, are the usual cause of this severe disorder (Herlitz-JEB). Rarely, a non-Herlitz JEB phenotype occurs which is non-lethal, owing to one LAMB3 allelic containing a mutation that preserves some residual β3 polypeptide and hence limited ability to assemble laminin-5.

In this issue, **Buchroithner *et al***[1] (p. 1279) report on an adult patient with non-Herlitz JEB, who was a compound heterozygote for LAMB3. A nonsense mutation on one allele, R635X, generated mRNA with a premature terminal codon subject to NMD. The second allele contained a 3009T→C mutation that did not alter codon translation (since both GGT and GGC code for the amino acid glycine), but instead introduced an exonic splice site in exon 20. Remarkably, the spliceosome used this new exonic splice site preferentially over the intact wild-type splice site at the exon 20:intron 20 border, despite the latter having a 'stronger' signal by established mathematical modeling algorithms. Moreover, the resultant aberrantly spliced LAMB3 mRNA contained a stop codon in exon 21, generating a premature termination codon (44 base pairs short of the normal termination codon). Despite the presence of premature termination codons in both allelic mRNAs, LAMB3 mRNA and polypeptide were still detectable in this patient's skin, owing to escape of the defective 3009T→C-derived mRNA from NMD, and the persistence of minute amounts of correctly spliced mRNA from the same allele. Besides reporting on a remarkable mutation in this specific skin disorder, these authors clearly demonstrate that exceptions to the proposed rules of pre-mRNA splicing and mRNA degradation can occur. Hence, exact RNA analysis is necessary to decipher pathogenetic events in genetic diseases; mathematical modeling of the consequences of mutations may be insufficient.

References

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Ocular fibrosis following injury: it's a Smad Smad world

Diverse cellular processes such as differentiation, migration, proliferation, and apoptosis are mediated via transforming growth factor-β (TGF-β) signaling pathways. Activation of type 1 TGF-β receptors (also called 'activin receptor-like kinases') results in phosphorylation of cytoplasmic receptor-regulated Smad proteins ('R-Smads' including Smads 1–3, 5, and 8). The latter form heterodimeric complexes with Smad4 and accumulate in the nucleus to regulate gene expression. Inhibitory Smads ('I-Smads' including Smad6 and Smad7) antagonize TGF-β signaling by competitively inhibiting the R-Smads and by promoting receptor ubiquitination and degradation. Dysregulation of TGF-β–Smad signaling has been implicated in human developmental disorders, neoplasia, autoimmune disease, and fibrosis.

In this issue, **Saika *et al***[1,2] (p. 1259; 1245) explore the effects of altered Smad signaling on two different aspects of ocular pathobiology in separate communications. The first report examines injury-induced epithelial–mesenchymal transition (EMT) of lens epithelial cells, which contributes to lens fibrosis. This type of injury has been observed in the human lens capsule following cataract surgery and can cause lens opacification, contraction, and visual impairment. Using an *in vivo* murine model, transient adenoviral transfer of inhibitory Smad7 cDNA prevented EMT in lens epithelial cells following experimental injury. Induction of Smad7 was associated with enhanced cell proliferation and abolished upregulation of *snail*, a zinc-finger-containing transcriptional repressor associated with suppression of the epithelial phenotype in EMT. This work provides *in vivo* evidence that Smad7 gene transfer could be used therapeutically to prevent or treat postoperative capsular fibrosis.

In a second report (p. 1245), the authors examine Smad signaling in retinal pigment epithelial cells (RPE) in an experimental model of proliferative vitreoretinopathy (PVR). In the model, RPE cells dedifferentiate and undergo EMT following retinal detachment leading to the formation of fibrous tissue and vitreous retraction. Smad3-knockout mice show markedly reduced accumulation of fibrous tissue in detached retinas compared to wild-type animals. The effect was attributed to blocking the EMT of RPE cells in the knockouts suggesting that Smad3 is required for fibrosis to occur in PVR associated with retinal detachment.

In addition to suggesting novel therapeutic options, these studies warrant keeping an 'eye' on TGF-β–Smad signaling in future investigations of ocular and other forms of fibrosis following injury.

References

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2 Saika S, Kono-Saika S, Tanaka T, *et al*. Smad3 is required for dedifferentiation of retinal pigment epithelium following retinal detachment in mice. Lab Invest 2004;84:1245–1258.