Japanese Society of Allergology. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure of potential conflict of interest: Y. Itagaki's, R. Teshima's, and K. Matsunaga's institution has received a Japanese Health and Labor Sciences Research Grant for this work. Y. Fukutomi's institution has received grants from the Ministry of Health, Labour and Welfare of Japan, and the Japan Agency for Medical Research and Development (15mk0101042) for this work. A. Fukushima has received board membership from Santen and consultancy fees from Kowa. Payment for lectures has been paid to the author and/or the author's institution by Alcon, Santen, Senju, Kowa, Kyowa-Kirin, Tanabe-Mitsubishi, Kissei, Ohtsuka, Abbvie, and Eisai; both the author and the author's institution have received payment for manuscript preparation from Alcon; the author has patents from Nidek. E. Noguchi's institution has received a grant from Grant-in-Aid for Scientific Research; is personally employed by the University of Tsukuba; and has received lectures fees from Otsuka Pharmaceutical. M. Nakamura is employed by HOYU Co. Ltd. H. Saito's institution has received a grant from the Ministry of Health, Labour & Welfare, Japan; is employed by the National Center for Child Health & Development, Japan; and has received payment for lectures from MSD (Merck Sharp and Dohme) K.K., Japan, Shiseido Co., Ltd, GlaxoSmithKline K.K., Japan, Kyowa Hakko Kirin Co., Ltd, and the World Allergy Organization. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online June 17, 2017.
http://dx.doi.org/10.1016/j.jaci.2017.03.019

Systems approach to uncover signaling networks in primary immunodeficiency diseases

To the Editor:

We describe here an approach to improve diagnoses and further our understanding of functional defects of primary immunodeficiency diseases (PIDs) using time-of-flight mass cytometry (CyTOF) to reveal the signaling of all circulating immune cells.

PIDs were historically diagnosed by a narrow, pathognomonic constellation of signs and symptoms. However, ever-broadening phenotypes have become apparent for diseases such as gain-of-function signal transducer and activator of transcription (STAT) 1. Moreover, distinct genetic mutations may share a single phenotype, especially if they share a signaling pathway (eg, LPS responsive beige-like anchor [LRBA] deficiency and cytotoxic T lymphocyte–associated antigen 4 haploinsufficiency). Thus, there has been an increasing reliance on genetic definitions of PIDs. However, sequencing cannot identify whether a novel mutation in a "known PID gene" will lead to a loss-of-function phenotype, a gain-of-function phenotype, or no phenotype at all. In this "postexome" era, identification of immune diseases would be greatly facilitated by a broad, unbiased *functional* analysis that parallels the broad, unbiased genetic analysis provided by next-generation sequencing.

This proof-of-concept study shows the potential of CyTOF to characterize a broad range of cells and signals. We began by testing the responses of circulating immune cells to canonical stimuli (cytokines and TLR agonists) in 5 healthy controls. Samples of whole blood were aliquoted and portions were stimulated with a cytokine or TLR agonists (IFN-α, IL-2, IL-5, IL-6, IL-7, IL-10, IL-17, IL-21, IL-25, LPS, and PMA) for 15 minutes; 1 aliquot was left unperturbed. We used CyTOF to measure more than 40 different markers simultaneously, including 9 intracellular phospho-proteins involved in signaling pathways (p38, ERK, PLCγ2, STAT1, STAT3, STAT5, S6 kinase, IκB, and AKT). We identified 18 types of circulating innate and adaptive immune cell types in the blood by gating (see Fig E1 in this article's Online Repository at www.jacionline.org) and examined phospho-signaling responses in these cell types at baseline and after stimulation (Fig 1; see Tables E1 and E2 in this article's Online Repository at www.jacionline.org). Examining responses after 15-minute stimulations minimized the impact of secondary signals that might arise at later time points.

This approach identified known patterns of stimuli and responses spanning both lymphoid and myeloid lineages including granulocytes, such as STAT5 in response to IL-2 and IL-7 and STAT3 in response to IL-6 and IL-10 (Fig 1). We noted that activated $CD4^+$ and $CD8^+$ T cells, respectively, had minimal or no increase in pSTAT5 in response to IL-7. In contrast, resting memory or naive T-cell lineages showed strong responses. These results can be explained by the reduced expression of IL-7 receptor in activated T cells.[1] Notably, IL-7R was not used in gating. Thus, our algorithm detected patterns of differential responses to IL-7 without an *a priori* understanding of IL-7R expression.

Hierarchical clustering indeed showed that functional signaling responses largely mirrored developmental lineages (see Fig E2 in this article's Online Repository at www.jacionline.org). Interestingly, we found that myeloid dendritic cells, plasmacytoid dendritic cells, and $CD16^+$ monocytes clustered with lymphoid cells, while $CD16^-$ monocytes clustered with myeloid cells. This grouping may reflect the functional propensity of $CD16^+$ monocytes to differentiate into dendritic cells.[2] These results show that even cells within the same developmental lineages may have varying degrees of responses to stimuli.

To demonstrate the utility of CyTOF in elucidating PIDs with broad phenotypes, we studied 2 patients with PID as a proof-of-principle. We started with an adolescent patient with chronic mucocutaneous candidiasis (CMC) identified with a monoallelic mutation in STAT1 (p.R274W), producing a GOF phenotype. CMC in these patients has been attributed to defective T_H17 immunity.[3] We first examined whether any *baseline* phosphorylation in our GOF STAT1 subject fell outside the 95% CI established in controls. At baseline, we unexpectedly found increased STAT3 phosphorylation in T cells (Fig 2, *A*). We did not find increased STAT1 phosphorylation at baseline, consistent with many previous studies. Next, we examined responses of the GOF STAT1 subject to stimuli as compared with controls (see Fig E3 in this

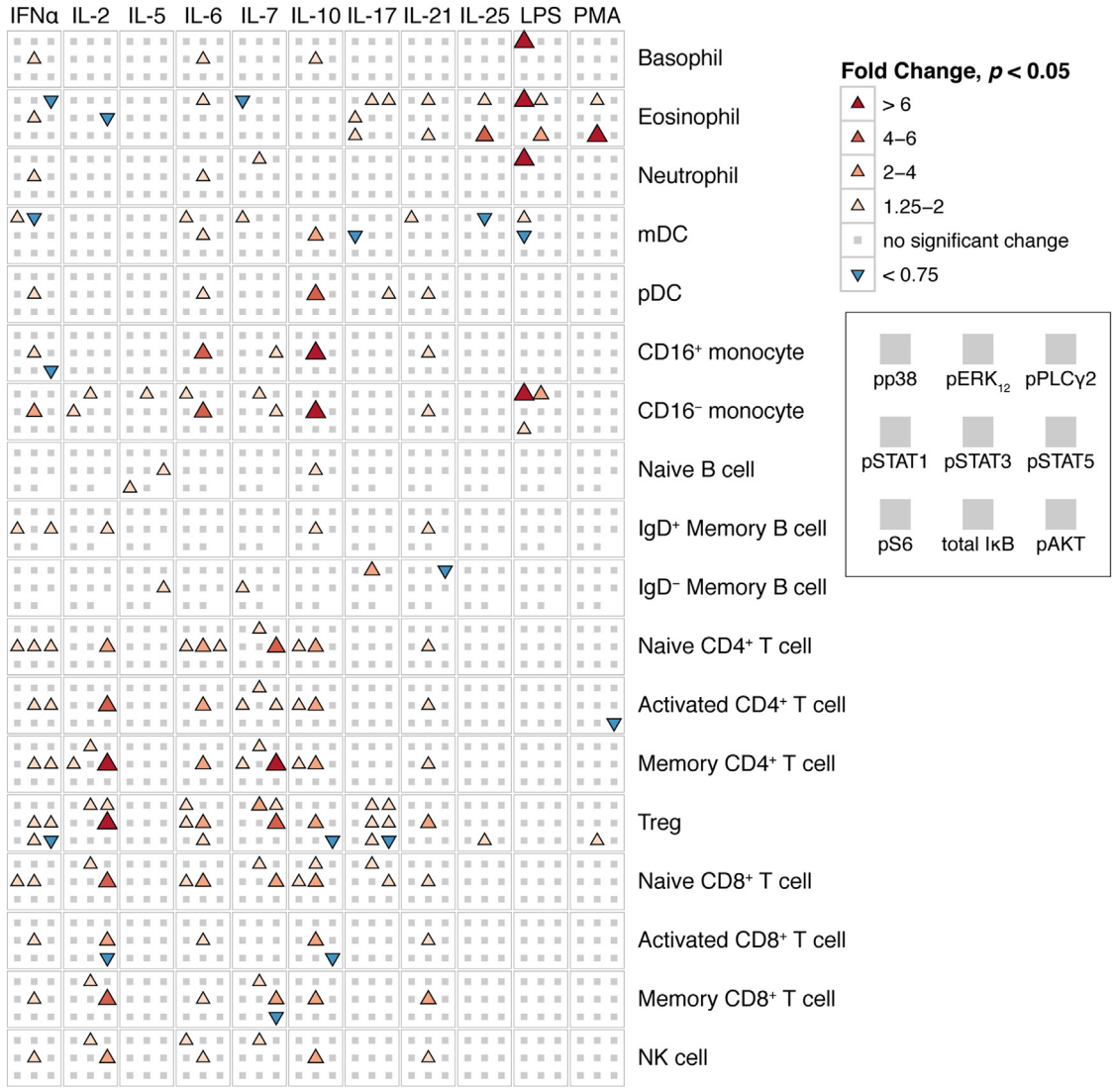

FIG 1. Signaling responses of immune cell subtypes to canonical stimuli. Fold changes of phospho signaling proteins after stimulation compared with baseline across 9 pathways, shown according to stimuli and cell subtype. All responses were measured 15 minutes after stimulation. This signaling map compiled from 5 healthy subjects provides a comprehensive view of well-established, recently described, and previously undescribed signaling changes. *mDC,* Myeloid dendritic cells; *NK,* natural killer; *pDC,* plasmacytoid dendritic cell.

article's Online Repository at www.jacionline.org). The increased baseline pSTAT3 we saw in T cells did not appear to necessarily affect signaling function, as the same cells largely had normal responsiveness to stimuli compared with controls. However, memory $CD4^+$ T cells did show decreased STAT3 responsiveness to IL-6 (Fig 2, *C*). Defective pSTAT3 response to IL-6 was also seen in $CD16^+$ monocytes (Fig 2, *C*) and may merit exploration to explain whether concurrence of inflammatory disorders in CMC may be secondary to this defective pathway. Activated $CD4^+$ T cells showed increased STAT3 responsiveness to IL-25. The IL-25/STAT3 pathway has been implicated in multipotent human mesenchymal stromal cells (hMSCs) suppressing T_H17 cell responses.[4] We did not look at hMSC signaling, but future studies could investigate the possibility that aberrant STAT1 and STAT3 signaling in hMSCs contributes to CMC. Another putative mechanism of impaired T_H17 immunity is increased pSTAT1 in response to STAT1- and STAT3-dependent cytokines.[5] In our GOF STAT1 subject, we saw increased STAT1 responsiveness to IL-10 in regulatory T (Treg) cells compared with controls (Fig 2, *C*). IL-10–induced STAT3 phosphorylation in Treg cells is known to suppress T_H17-mediated inflammation.[6] As a cytokine that activates both STAT1 and STAT3, IL-10 in these subjects may enhance this T_H17 cell suppression, leading to CMC.

We next examined a subject with hyper-IgE syndrome (HIES) due to an autosomal-dominant STAT3 mutation (p.R382W). Low phosphorylation of STAT3 by IL-6 and deficiency of IL-17A– and IL-22–producing T cells have been well-documented signaling abnormalities, but the mechanisms underlying all the phenotypic features of HIES have not been fully elucidated. Compared with controls, we unexpectedly found increased baseline pSTAT1 in multiple cells (Fig 2, *B*).

In response to stimuli, we found diminished STAT3 responsiveness to IL-6 in this patient with HIES, prominently observed in neutrophils (Fig 2, *D*; see Fig E4 in this article's Online Repository at www.jacionline.org). The most striking aberrancy in the