Title
Systems approach to uncover signaling networks in primary immunodeficiency diseases

Journal
Journal of Allergy and Clinical Immunology, 140(3)

ISSN
0091-6749

Authors
Choi, Jeff
Fernandez, Rosemary
Maecker, Holden T
et al.

Publication Date
2017-09-01

DOI
10.1016/j.jaci.2017.03.025

Peer reviewed

HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

J Allergy Clin Immunol. 2017 September ; 140(3): 881–884.e8. doi:10.1016/j.jaci.2017.03.025.

Systems Approach to Uncover Signaling Networks in Primary Immunodeficiency Diseases

Jeff Choi, B.Sc.[1], **Rosemary Fernandez, Ph.D.**[2], **Holden T. Maecker, Ph.D.**[2], and **Manish J. Butte, M.D., Ph.D.**[3,*]

[1]School of Medicine, Stanford University, Stanford, California 94305, USA

[2]Human Immune Monitoring Core, Stanford University, Stanford, California 94305, USA

[3]Department of Pediatrics, Division of Immunology, Allergy, and Rheumatology, University of California, Los Angeles, California 90095, USA

Capsule Summary

This broad, unbiased approach of studying signaling across all circulating immune cells in healthy subjects allowed identification of disrupted signaling networks in patients with primary immunodeficiencies.

Keywords

CyTOF; Immunodeficiency; Signaling

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 like gain-of-function STAT1. Moreover, distinct genetic mutations may share a single phenotype, especially if they share a signaling pathway (e.g., LRBA deficiency, CTLA-4 haploinsufficiency). Thus, there has been an increasing reliance on genetic definitions of

*To whom correspondence should be addressed: mbutte@mednet.ucla.edu, Division of Immunology, Allergy, and Rheumatology, Department of Pediatrics, 10833 Le Conte Avenue, 12-430 MDCC, Los Angeles, CA 90095, 310-825-6481, 310-825-9832 (fax).
[3]Current affiliation Department of Pediatrics, Division of Immunology, Allergy, and Rheumatology, University of California, Los Angeles. mbutte@mednet.ucla.edu manish.butte@stanford.edu

Author Contributions

Conceptualization, Software, Investigation, Visualization, Writing, J.C. and M.J.B. Funding and Supervision, M.J.B.

Competing interests: All authors declare no conflicting financial interest related to this study.

Data and materials availability: R scripts written to analyze data are available from the corresponding author.

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 "post-exome" 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 five healthy controls. Samples of whole blood were aliquoted and portions were stimulated with a cytokine or TLR agonists (IFNα, IL-2, IL-5, IL-6, IL-7, IL-10, IL-17, IL-21, IL-25, LPS, and PMA) for 15 minutes; one aliquot was left unperturbed. We employed CyTOF to measure over 40 different markers simultaneously, including nine intracellular phospho-proteins involved in signaling pathways (p38, ERK, PLCγ2, STAT1, STAT3, STAT5, S6 kinase, IκB, and AKT). We identified eighteen types of circulating innate and adaptive immune cell types in the blood by gating (Fig. S1) and examined phospho-signaling responses in these cell types at baseline and after stimulation (Fig. 1 and Tables S1 and S2). 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 naïve 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 (Fig. S2). Interestingly, we found that mDC, pDC, 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 DCs[2]. These results show how 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 two PID patients 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 Th17 immunity[3]. We first examined whether any *baseline* phosphorylation in our GOF STAT1 subject fell outside the 95% confidence interval established in controls. At baseline, we unexpectedly found increased STAT3 phosphorylation in T cells (Fig. 2A). 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 (Fig. S3). 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 to controls. However, memory $CD4^+$ T cells did show decreased STAT3 responsiveness to IL-6 (Fig. 2C). Defective pSTAT3 response to IL-6 was also seen in $CD16^+$ monocytes (Fig. 2C) 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 (hMSC) suppressing Th17 cell responses[4]. We did not look at hMSC signaling but future studies could investigate the possibility that aberrant STAT1 and STAT3 signaling in hMSC contributes to CMC. Another putative mechanism of impaired Th17 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 Tregs compared to controls (Fig. 2C). IL-10-induced STAT3 phosphorylation in Tregs is known to suppress Th17-mediated inflammation[6]. As a cytokine that activates both STAT1 and STAT3, IL-10 in these subjects may enhance this Th17 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 to controls, we unexpectedly found increased baseline pSTAT1 in multiple cells (Fig. 2B).

In response to stimuli, we found diminished STAT3 responsiveness to IL-6 in this HIES patient, prominently observed in neutrophils (Fig. 2D and Fig. S4). The most striking aberrancy in the STAT3 deficiency subject was the decrease, especially in eosinophils, of pSTAT3 in response to IFNα, IL-10, and IL-17 (Fig. 2D), stimuli that normally increase STAT3 phosphorylation. A similar reversal of STAT3 activity in response to IFNα was seen in neutrophils (Fig. S4). We noted that IFNα is occasionally used to treat hypereosinophilic syndromes and has been implicated in suppressing eosinophils through multiple modalities[7]. We advance the idea here that eosinophils could underlie some symptoms of HIES because of decreased suppressibility. IFNα has been shown to stimulate release of TRAIL from neutrophils to induce apoptosis in leukemic cells[8]. If this effect depends on STAT3, this finding could help explain the link between HIES and increased incidence of lymphoma.

We note here the preliminary but intriguing finding that the subject with GOF STAT1 had elevated baseline STAT3 activity while STAT3 deficiency subject had elevated baseline STAT1 activity. Our findings of baseline dysregulation may be reflective of a compensatory mechanism across a signaling network. Indeed, a recent study showed that STAT3 and STAT1 work together to drive and specify transcriptional outputs, respectively[9]. We hypothesize that in GOF STAT1 and STAT3 deficiency, cross-talk between these STAT signals may be a compensatory response.

In summary, CyTOF analyses of healthy controls recapitulated established signaling responses and identified interesting patterns for subsequent exploration. CyTOF-based analysis of more controls –using any combination of pathways, cell subtypes, and stimuli of immunological interest– can build reliable maps showing primary signaling responses

expected in healthy individuals of all ages. As we showed, these maps can serve as comparisons for CyTOF data from patients, to both validate known signaling patterns and provide a data mine of previously unknown signaling defects to complete a mechanistic picture of the disease or prioritize pathways for validation. Moreover, CyTOF signaling maps of healthy controls themselves may fuel a wealth of investigative research, such as by studying differences due to aging or due to gender. We propose that the broad pattern of signaling responses can be used as functional fingerprints of immune cell subtypes much like cell-surface markers are phenotypic fingerprints. Defining PIDs by a combination of hallmark signals as well as genetic defects could help explain both commonalities across what might otherwise be considered distinct diseases as well as differences within a single genetic disease.

Online Materials and Methods

Whole blood from controls and subjects were drawn after informed consent and approved by Stanford Institutional Review Board. Controls were healthy adults (20s–40s) at the time of sampling, on no immunomodulatory medicines and without known PID. Patient 1 is a 17 year old with documented GOF STAT1 with characteristic phenotypes and Patient 2 was an 18 year old with documented STAT3 deficiency. Both subjects were clinically well at the time of blood collection. Whole blood samples were aliquoted into portions (0.2 mL each), and stimulated with either cytokines, TLR agonists, PMA & ionomycin, or control. Treated cells were surface stained, fixed, permeabilized for 15 minutes, and stained for phospho-proteins as described previously[10]. CyTOF surface markers for gating and intracellular phospho-proteins were conjugated with metals according to manufacturer's directions (Table S3). Gating for identification of immune cell subtypes was performed in Cytobank[11]. All analyses were performed in R[12]. Gates containing less than 0.0001% of events on any date were removed. Events with phospho-signal values greater than 0 were considered for analysis. Raw phospho-signal values were adjusted to allow comparison of phosphorylation across the five control CyTOF experiments run on five experiment dates as follows. The median baseline (unstimulated) phosphorylation values for each signaling pathway-cell type-experiment date combination was calculated. Adjustment factors were next calculated to align every experiment date's values to the bootstrapped mean values for each signaling pathway-cell type combination. Following this adjustment, all controls had the same baseline values for each signaling pathway-cell type combination.

The same adjustment factors calculated for signaling pathway-cell type combination in each control was applied to post-stimulus signaling values. This allowed comparison of post-stimulation phospho-signaling fold changes from baseline across the five experiment dates. For significance testing, we calculated the bootstrapped means for each cell type-stimulus-signaling pathway combinations across controls to derive the 95% confidence interval, and calculated p values using permutation testing. CyTOF on the two PID subjects were run on two of the five aforementioned experiment dates. To compare phospho-signaling differences in PID subjects and all controls, we applied the same adjustment factors calculated for signaling pathway-cell type combinations of the control run on the same date as to the corresponding PID subject. To determine if PID subjects had aberrant responses to stimuli, we gathered all fold changes that lay outside the 95% confidence interval of corresponding

cell type-stimulus-signaling pathway fold changes in controls with p-values of less than 0.05. Hierarchical clustering of cell-stimulus-pathway fold change combinations was performed using the complete-linkage method in R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. T. Thauland for helpful comments. We acknowledge funding from the NIH; the Stanford Institute for Immunity, Transplantation, and Infection; and the Stanford Child Health Research Institute.

Abbreviations

PID	(primary immunodeficiency diseases)
CyTOF	(time-of-flight mass cytometry)
GOF	(gain of function)
CMC	(chronic mucocutaneous candidiasis)

References

1. Xue HH, Kovanen PE, Pise-Masison CA, Berg M, Radovich MF, Brady JN, et al. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. Proc Natl Acad Sci U S A. 2002; 99:13759–64. [PubMed: 12354940]
2. Randolph GJ, Sanchez-Schmitz G, Liebman RM, Schakel K. The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. J Exp Med. 2002; 196:517–27. [PubMed: 12186843]
3. Ng WF, von Delwig A, Carmichael AJ, Arkwright PD, Abinun M, Cant AJ, et al. Impaired T(H)17 responses in patients with chronic mucocutaneous candidiasis with and without autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Allergy Clin Immunol. 2010; 126:1006–15. 15 e1–4. [PubMed: 20934207]
4. Wang WB, Yen ML, Liu KJ, Hsu PJ, Lin MH, Chen PM, et al. Interleukin-25 Mediates Transcriptional Control of PD-L1 via STAT3 in Multipotent Human Mesenchymal Stromal Cells (hMSCs) to Suppress Th17 Responses. Stem Cell Reports. 2015; 5:392–404. [PubMed: 26321145]
5. Liu L, Okada S, Kong XF, Kreins AY, Cypowyj S, Abhyankar A, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. J Exp Med. 2011; 208:1635–48. [PubMed: 21727188]
6. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity. 2011; 34:566–78. [PubMed: 21511185]
7. Morita M, Lamkhioued B, Soussi Gounni A, Aldebert D, Delaporte E, Capron A, et al. Induction by interferons of human eosinophil apoptosis and regulation by interleukin-3, granulocyte/macrophage-colony stimulating factor and interleukin-5. Eur Cytokine Netw. 1996; 7:725–32. [PubMed: 9010674]
8. Tecchio C, Huber V, Scapini P, Calzetti F, Margotto D, Todeschini G, et al. IFNalpha-stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. Blood. 2004; 103:3837–44. [PubMed: 14726404]

9. Hirahara K, Onodera A, Villarino AV, Bonelli M, Sciume G, Laurence A, et al. Asymmetric Action of STAT Transcription Factors Drives Transcriptional Outputs and Cytokine Specificity. Immunity. 2015; 42:877–89. [PubMed: 25992861]

10. Fernandez, R., Maecker, H. Cytokine-Stimulated Phosphoflow of Whole Blood Using CyTOF Mass Cytometry. Bio-protocol. 2015. http://www.bio-protocol.org/e1495

11. Kotecha N, Krutzik PO, Irish JM. Web-based analysis and publication of flow cytometry experiments. Curr Protoc Cytom. 2010 Chapter 10:Unit10 7.

12. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing; 2014.

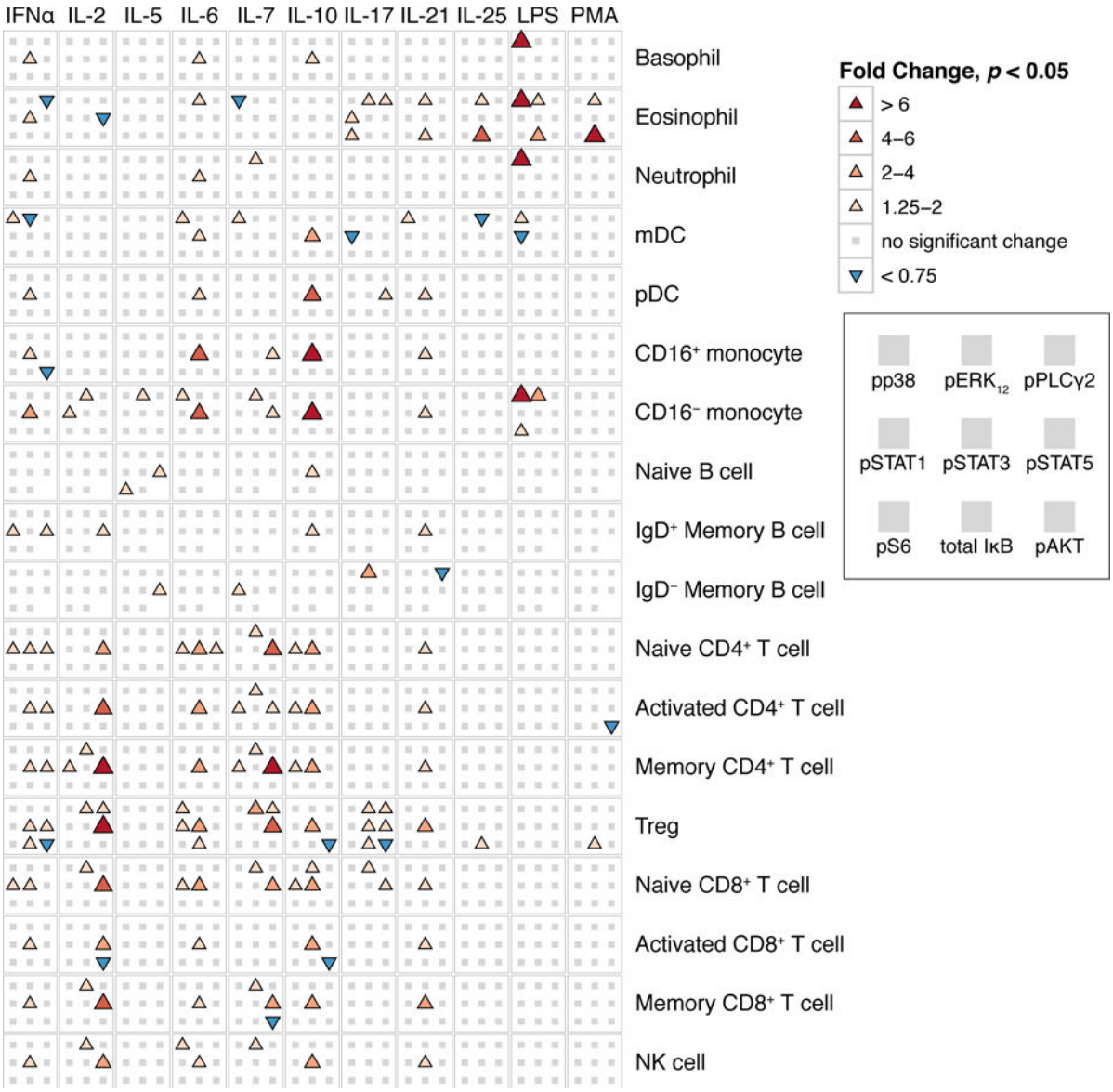

Fig.1. Signaling responses of immune cell subtypes to canonical stimuli

Fold changes of phospho signaling proteins after stimulation compared to baseline across nine pathways, shown according to stimuli and cell subtype. All responses were measured 15 minutes after stimulation. This signaling map compiled from five healthy subjects provides comprehensive view of well-established, recently described, and previously undescribed signaling changes.

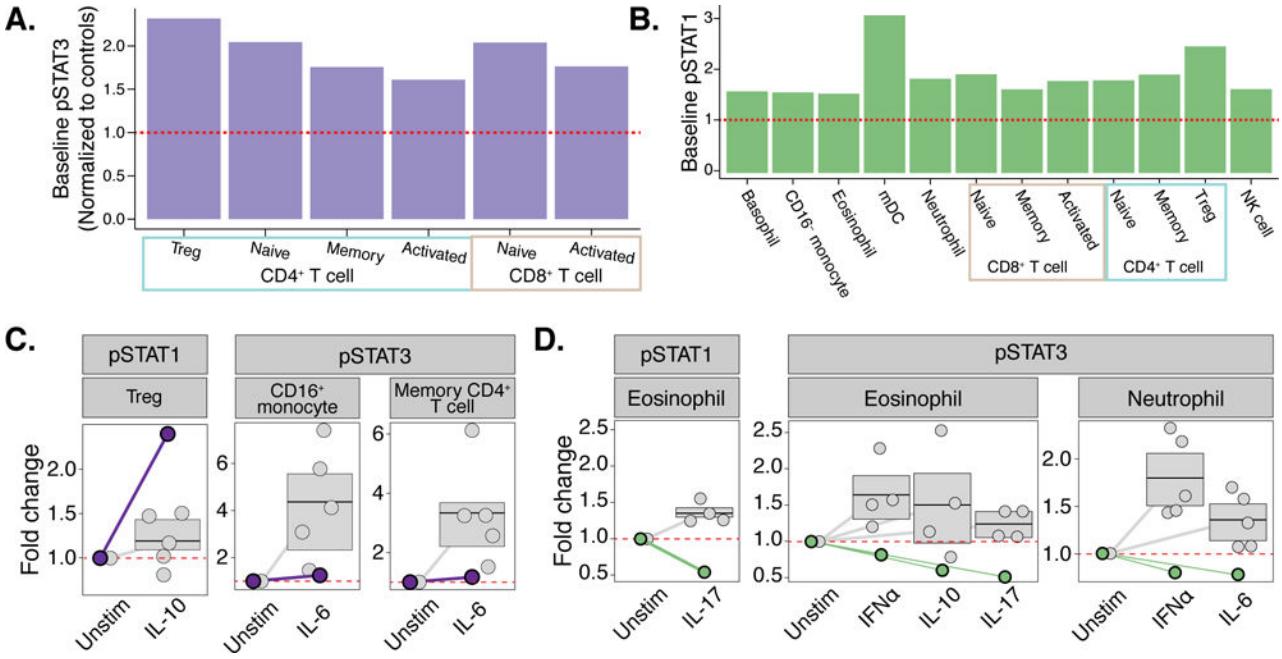

Fig. 2. Defects in baseline signals and responses to signals in PI disease

Baseline phosphorylation levels of subjects with (***A***) GOF STAT1 and (***B***) STAT3 deficiency that are greater than 1.5 fold or less than 0.75 fold of corresponding baseline phosphorylation levels in controls. Signaling fold changes in (***C***) GOF STAT1 and (***D***) STAT3 deficiency subjects that fall outside of the 95% CI of corresponding fold changes in controls (indicated by boxes) with p value of <0.05 are shown. Unstimulated points are scaled to the unstimulated value for the subject with GOF STAT1 (***C***) or STAT3 deficiency (***D***) or the average for the five healthy subjects. Stimulation responses are shown for the PID subjects (purple or green circle) and the healthy controls (grey circles). Stimuli and signals are across the top, and cell type is across the side. Only the responses that are statistically significant are shown.