

Insights into primary immune deficiency from quantitative microscopy

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Recent advances in genomics-based technology have resulted in an increase in our understanding of the molecular basis of many primary immune deficiencies. Along with this increased knowledge comes an increased responsibility to understand the underlying mechanism of disease, and thus increasingly sophisticated technologies are being used to investigate the cell biology of human immune deficiencies. One such technology, which has itself undergone a recent explosion in innovation, is that of high-resolution microscopy and image analysis. These advances complement innovative studies that have previously shed light on critical cell biological processes that are perturbed by single-gene mutations in primary immune deficiency. Here we highlight advances made specifically in the following cell biological processes: (1) cytoskeletal-related processes; (2) cell signaling; (3) intercellular trafficking; and (4) cellular host defense. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Primary immune deficiency, microscopy, cell biology, cytoskeleton, host defense

In recent years, the field of primary immune deficiency (PID) has benefited tremendously from the explosion in technology resulting from the advent of relatively inexpensive and increasingly sophisticated sequencing techniques. To date, more than 5000 mutations have been identified leading to 248 unique PIDs (<http://web16.kazusa.or.jp/rapid/>). With the discovery of the genetic basis of these immunologic diseases comes the opportunity to use samples from these naturally occurring deficiency “models” to address important questions in cell biology and at the same time gain true insight into the way human immunity works on a mechanistic level. In the best case these insights can help improve both the diagnosis and treatment of these often rare, devastating, and complex diseases.

In parallel with genetic technology, the field of optical imaging has undergone a rapid revolution in the past 10 years through use of highly quantitative microscopy techniques. This has led to an extraordinary period of growth in the field of cell biology, as well as objective answers to many questions previously considered purely theoretical. A variety of optical microscopy approaches

Abbreviations used

AP3: Adaptor protein 3
Arp2/3: Actin-related proteins 2 and 3
CDC42: Cell division cycle 42
CGD: Chronic granulomatous disease
CHS: Chediak-Higashi syndrome
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
DOCK8: Dedicator of cytokinesis 8
FHL: Familial hemophagocytic lymphohistiocytosis
HPS2: Hermansky-Pudlak syndrome type 2
IS: Immunologic synapse
LAD-I: Leukocyte adhesion deficiency type 1
LFA-1: Lymphocyte function-associated antigen 1
LYST: Lysosomal trafficking regulator
MAGT1: Magnesium transporter 1
MTOC: Microtubule organizing center
NADPH: Nicotinamide adenine dinucleotide phosphate
NET: Neutrophil extracellular trap
NF- κ B: Nuclear factor κ B
NIK: NF- κ B-inducing kinase
NK: Natural killer
PID: Primary immune deficiency
ROS: Reactive oxygen species
SNARE: Sensitive factor attachment protein receptor
TCR: T-cell receptor
TEM: Transmission electron microscopy
WAS: Wiskott-Aldrich syndrome
WASp: Wiskott-Aldrich syndrome protein

have enabled improvements in molecular localization, optical sectioning, 3-dimensional reconstruction, live cell imaging and even 4-dimensional reconstructions (Table 1).¹⁻³⁸ One of these technical advances, superresolution imaging, is particularly noteworthy because it has dramatically decreased the size of resolvable objects within cells from previous limits of about 200 nm to new reaches of even single-digit nanometer levels through the use of optics. Recognition for this powerful advance was provided through the 2014 Nobel Prize in Chemistry and Physics.

The application of highly quantitative imaging, objective image analysis, and, more recently, superresolution approaches to immune cell function has resulted in an increased understanding of the molecular basis of immunity, which has in turn led to a greater understanding of the diseases that occur as a result of perturbations of the immune system. Quantitative microscopy, when thoughtfully and carefully applied, provides detailed and powerful information about the subcellular localization of proteins of interest with relatively minimal biological sample input, making it a valuable tool for those seeking to study rare

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Disclosure of potential conflict of interest: J. S. Orange has received research support from CSL Behring. E. M. Mace declares that she has no relevant conflicts of interest.

Received for publication March 10, 2015; accepted for publication March 30, 2015.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2015.03.049>

TABLE I. High-resolution imaging techniques used in cell biology studies of PIDs, including references to use in PID

Name	Description	PID references
Confocal microscopy	Use of spatially aligned pinhole to improve resolution of fluorescence microscopy through exclusion of out-of-focus light	4-6, 8, 9, 12, 13, 15-22, 24-34, 36, 37
Superresolution microscopy	Use of optics or mathematic algorithms to achieve resolution below the diffraction limit of light	14, 15
Fluorescence wide field	Use of whole-specimen illumination to visualize structures labeled with fluorophores	10, 11, 35
TEM	Use of a focused beam of electrons passed through an ultrathin specimen to illuminate structural elements	1-3, 7, 15, 23, 25, 27, 34, 37, 38
Total internal reflection microscopy	Use of an evanescent wave to selectively illuminate membrane proximal 150 nm of the cell	24
Imaging flow cytometry	Combination of conventional flow cytometry with fluorescence microscopy to assess both population-based phenotype and protein localization	
Correlative light electron microscopy	Use of light microscopy of fixed cells, followed by imaging of structures by means of electron microscopy	24

human disorders, often in children from whom samples can be scarce.

Here we review the application of highly quantitative imaging to the field of PIDs, with a specific focus on 4 areas that have particularly benefited from this increased resolution, namely mutations that affect (1) cytoskeletal-related processes, (2) cell signaling, (3) intercellular trafficking, and (4) cellular host defense.

DEFECTS IN CYTOSKELETON-RELATED PROCESSES

The structural framework contained within the cell cortex is made up of filaments of cytoskeletal actin, also known as F-actin. Each cell contains robust F-actin rearrangement machinery that is required to rapidly change shape, generate force, reorient signaling complexes, and coordinate migration. The nucleation of F-actin is the process in which the building of an actin filament is begun and is critical for immune cell migration and effector function.³⁹ As such, mutations affecting the cytoskeleton have a profound effect and lead to significant disease.³⁹ In particular, the regulated control of F-actin nucleation and remodeling is required for the formation and function of the immunologic synapse (IS; Fig 1, A). The IS can be broadly described as a highly specialized signaling platform formed between 2 immune cells and was originally described between a CD4⁺ T cell and an antigen-presenting cell. Arguably one of the best-characterized types of IS, however, might be the so-called “lytic synapse,” which leads to the directed secretion of specialized secretory lysosomes or lytic granules. Both cytotoxic T lymphocytes (CTLs) and natural killer (NKs) cells form lytic synapses and seem to share many common mechanisms of synapse formation. Lytic synapse function occurs in 3 stages (recognition, effector, and termination), which can be further broken down into discrete steps.⁴⁰ Central to an increasingly appreciated number of these steps is the highly regulated function of the cytoskeleton (Table II),^{39,41,42} particularly F-actin nucleation, remodeling, and deconstruction. The central role of F-actin in immune cell function and its large-scale perturbation in the context of immune deficiency have been well reviewed elsewhere.³⁹

The study of PIDs affecting the regulation of F-actin have led to critical insight into the cytoskeletal processes of immune cells.

Here we focus specifically on those PIDs that have driven discoveries enabled by the use of quantitative microscopy. Of these, perhaps the best characterized and studied is Wiskott-Aldrich syndrome (WAS). More than 300 distinct mutations in Wiskott-Aldrich syndrome protein (WASp) have been described, leading to classical WAS, X-linked thrombocytopenia, or X-linked neutropenia.⁴³ WAS as a heritable condition was first described in 1954 and is recognized clinically by the characteristic triad of thrombocytopenia, neutropenia, and immune deficiency.⁴³ The WASp gene was identified in 1994 and led to the subsequent characterization of a large related family of proteins of the WASp family: N-WASp, WAVE, SCAR, WASF1-3, WASH, WHAMM, and JMY.⁴⁴ WASp (and its homologues) activates the actin-related proteins 2 and 3 (Arp2/3) complex to promote branched F-actin nucleation. This allows for a new filament of actin to be built at an angle to an existing filament, which provides tremendous structural integrity akin to interlocking girders in a building. Although actin nucleation is just one of many processes regulating F-actin dynamics (others include elongation, capping, severing, and linear nucleation), it is a critical component of migration, adhesion, and signaling. Because of this and WASp's ubiquitous expression in hematopoietic cells, WAS affects multiple immune cell types and functions.⁴³

WAS was one of the first PIDs to be studied from a cell biology perspective using higher-resolution microscopy. Scanning electron microscopy was initially used to study lymphocytes from patients with WAS¹ and semiquantitatively score the presence and relative size of microvillus projections on the cell surface. The morphologic abnormalities observed in patients' lymphocytes were the first indication that cytoskeletal structural alterations were contributing to the loss of immune function, although at the time, the mechanism was not clear. Subsequent studies also described the “bald” phenotype of WAS lymphocytes^{2,3,45} through electron microscopy with regard to microvillus projections. Certain studies have suggested that WASp deficiency does not alter lymphocyte microvilli frequency or length, but these have often been done under activating conditions that could potentially access pathways bypassing WASp deficiency.^{1,3,45} Furthermore, the effects seen in cells from patients with WAS can be variable, including some “baldness” but also altered and heterogeneous morphology, especially in T-cell lines. B cells

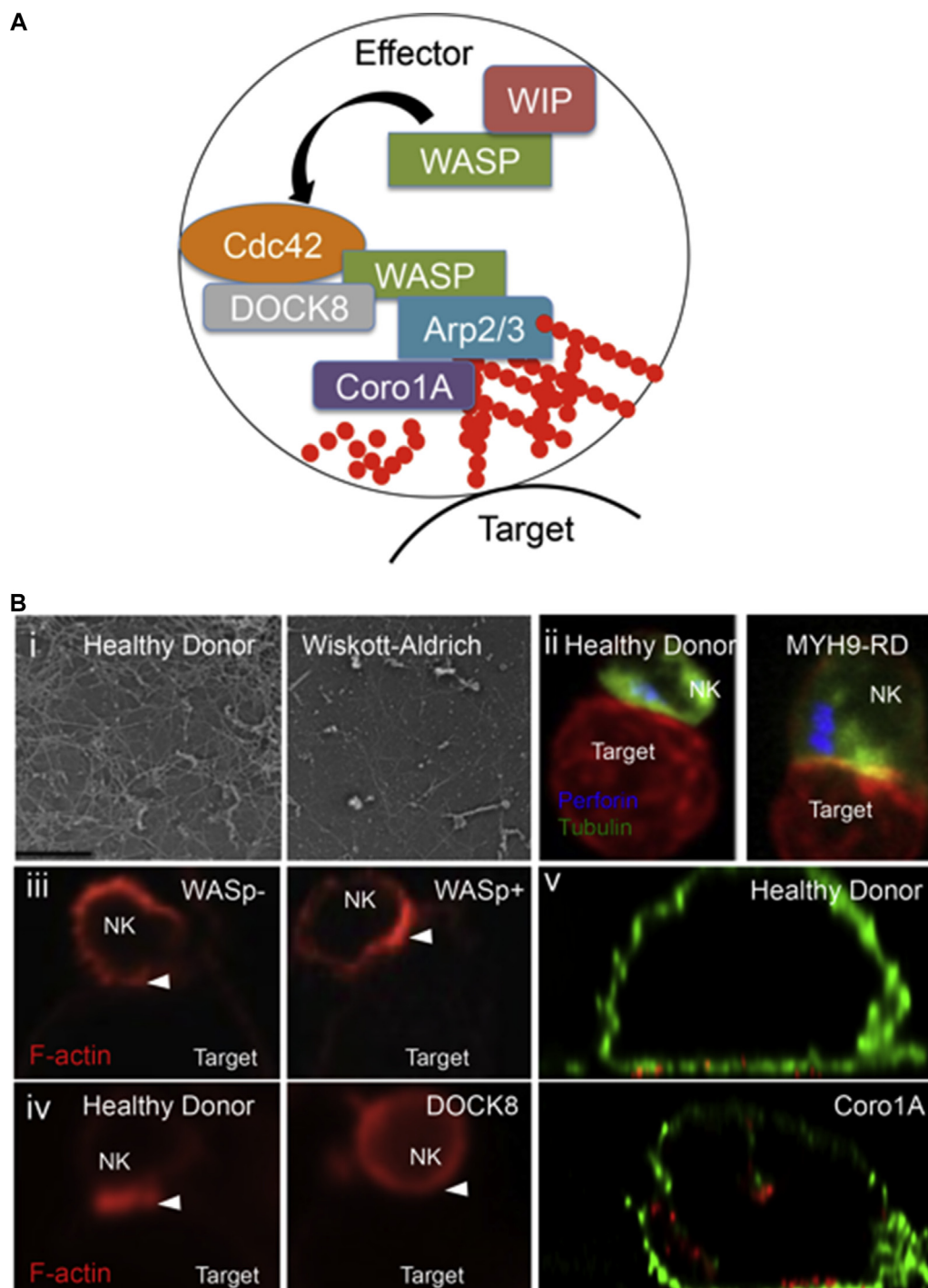


FIG 1. Effect of PIDs on the actin cytoskeleton. **A**, Pathway leading to F-actin polymerization and structural rearrangement at the immune synapse. On activation, autoinhibition of WASp by WIP is relieved. DOCK8 promotes both WASp recruitment and CDC42 function. WASp enables activation of Arp2/3, which forms *de novo* branched actin filaments that are required for firm adhesion and receptor clustering at the immune synapse. Existing branched filaments undergo depolymerization and/or rearrangement by Coronin 1A (Coro1A). **B**, Effect of PIDs affecting the actin cytoskeleton, as measured by using high-resolution microscopy. *i*, Transmission electron micrograph of F-actin at the NK cell immune synapse from a healthy donor (left) or patient with WAS (right). Note the absence of F-actin filaments and lack of branched network as a result of the loss of WASp-driven Arp2/3 function. *ii*, Lytic granule polarization in NK cells from a healthy donor (left) or myosin IIA-deficient cell line (right) conjugated to susceptible target cells. Patients with MYH9-related disease (MYH9-RD) have NK cell deficiency because of an inability of NK cells to appropriately polarize to the immune synapse and subsequently degranulate and lyse virally infected target cells. Conjugates were fixed and stained for perforin (blue) or tubulin (green). *iii*, F-actin accumulation at the immune synapse formed between a WASp-deficient (left) or WASp-expressing (right) NK cell isolated

TABLE II. Key cellular structures affected by PIDs

Structure	Description	Size	Reference (review)
MTOC	Centrosome; facilitates delivery of lytic granules to immune synapse		42
F-actin	Smallest cytoskeletal filament; promotes adhesion and migration	5-10 nm	39
Microtubules	Largest cytoskeletal filament; framework for transport of lytic granules and other cargo	15-20 nm	42
Lytic granules	Specialized acidified lysosomes containing perforin and granzymes	100-750 nm	41

from WASp-deficient mice showed some microvillus defects,³ but these experiments also used *in vitro* cellular activation. The cell-surface morphology of NK cells from patients with WAS has never been specifically examined by using electron microscopy. In light of the divergent response of NK cells from T cells in regard to IL-2-induced actin polymerization (see below), it would be of interest to determine whether WAS NK cells have more profound microvillus defects at baseline.

Cloning of the WAS gene⁴⁴ led to identification of cell division cycle 42 (CDC42) as the target of WASp control⁴⁶ and a greater understanding of the role of WASp in the regulation of the actin cytoskeleton. Despite this clear mechanistic linkage, the specific demonstration of this biology in T cells was not easily apparent. It was the quantitative confocal fluorescent imaging of NK cells that demonstrated that the defect in the formation and function of the actin-directed IS is what leads to loss of NK cell effector function in patients with WAS. Loss of WASp function leads to impaired F-actin accumulation at the NK cell IS and accompanying loss of perforin polarization.⁴ This observation provided a direct link between WASp function, synaptic F-actin, and the F-actin-driven NK cell polarization that is required for cytotoxicity.

The role for WASp in overarching IS function was extended by studies of the CD8⁺ CTL synapse. Interestingly, the study of the WAS CTL synapse highlighted key differences between NK cell and T-cell effector function with regard to the WASp-actin machinery. As opposed to NK cells, in which WASp is indispensable for complete adhesion to target cells and subsequent lytic granule polarization, WASp-deficient CTLs polarize lytic granules and the microtubule organizing center (MTOC) normally as determined by using confocal microscopy. Also, in contrast to WASp-deficient NK cells, CTLs degranulate at a frequency comparable with that of cells from healthy donors.⁵

Although no difference in the frequency of conjugate formation between CTLs from patients with WAS and target cells was specifically noted, it was proposed that the CTL synapse might be destabilized in the absence of WASp. In support of this hypothesis, naive CD4⁺ T cells from WASp^{-/-} mice have been shown to be incapable of stable synapse formation after migration.^{5,6} Interestingly, the CD4⁺ T-cell synapse from cell lines derived from patients with WAS showed abnormal F-actin protrusions and morphology but demonstrated normal conjugation efficiency with antigen-presenting cells. Three-dimensional reconstruction confocal microscopy showed a

decrease in the surface area of CD4⁺ T-cell synapses from patients with WAS formed with antigen-presenting cells. As in NK cells, there was also a lack of MTOC polarization to the synapse. This abnormality in T cells from patients with WAS was accompanied by defects in receptor accumulation at the synapse and abrogated Ca²⁺ signaling.

The use of time-lapse confocal microscopy added a new dimension to these investigations because it demonstrated that initial T-cell receptor (TCR) accumulation and phosphorylation of tyrosine residues at the synapse was normal but then destabilized over time. For the first time, this suggested an inability for T cells from patients with WAS to maintain TCRs clustered at the synapse. In addition, kinetic imaging showed that although the initial wave of Ca²⁺ signaling was intact, it could not be maintained. Together, these new insights support the hypothesis that the primary defect in CTLs from patients with WAS might be an inability to form stable synapses with the prolonged and sustained signaling needed for function.

Importantly, IL-2 has been observed to partially ameliorate the defect in NK cell cytotoxicity seen in cells from patients with WAS.^{4,7,47} This was initially defined as a macroscopic observation in ⁵¹Cr-release functional assays and subsequently advanced microscopically. These later studies demonstrate that IL-2 activates the WASp homolog WAVE2 independently of WASp, which can then restore F-actin accumulation, granule polarization, and cytotoxic function.⁷ This novel WASp-independent signaling pathway downstream of IL-2 was identified by using both live and fixed cell confocal microscopy and quantitatively demonstrated that IL-2 promoted *de novo* actin accumulation in the absence of WASp function. This was an unexpected insight into the differential actin polymerization in NK cells compared with T cells, in which WAVE2 is accessed constitutively for normal F-actin function.⁴⁸

In addition, platinum rotary shadowing transmission electron microscopy (TEM) was used in this work to visualize NK cells that had been “unroofed” at their synaptic interface to truly demonstrate the architecture of their cytoskeletal cortex. This approach, which allowed for the study of platinum replicas of the NK cell synapse, provides striking measurement of the degree of disorder in the absence of WASp function (Fig 1, B). Although this technique provided unparalleled levels of resolution in imaging the immune synapse, it does not distinguish among the different components present at the cell cortex; that said, the

from the peripheral blood of a patient with WAS treated with lentiviral gene therapy. Note the restored F-actin accumulation at the immune synapse (white arrow) that accompanies restored WASp expression. Conjugates were fixed and stained for F-actin (red). iv, Defective F-actin accumulation at the immune synapse (white arrow) in NK cells isolated from a DOCK8-deficient patient when compared with a healthy donor (left). Conjugates were fixed and stained for F-actin (red). v, Impaired lytic granule access to the plasma membrane in the absence of Coro1A function in a Coro1A-KD human NK cell line (bottom) compared with the control line (top). Cells were fixed and stained for F-actin (green), and lytic granules (red) were then visualized in the XZ plane by using stimulated emission depletion microscopy.

architecture of actin filaments are easily discerned and even measured.

The new insights into IL-2 in inducing WAVE2 function in NK cells from patients with WAS has led to a promising and presently active clinical trial to study the application of subcutaneous IL-2 injections in patients with WAS (NCT00774358). The rationale for IL-2 application in patients with WAS is that there could be benefit for restoration of NK cell function given the susceptibility of patients to herpesvirus infections and hematologic malignancies. The use of confocal microscopy to quantify the cytotoxic function of NK cells from patients after *in vivo* administration of IL-2 has provided some early support to the biochemical data in that there was a significant increase in basal levels of F-actin in NK cells from patients,⁷ as well as restoration of polarization of the cytolytic machinery toward the target cell.

A second novel approach for the treatment of WAS is the use of gene therapy. Autologous CD34⁺ hematopoietic stem cells were transduced with the full-length WAS gene initially through use of a retroviral vector⁸ and, subsequently, a lentiviral vector by using the endogenous WASP promoter.⁹ In the earlier work confocal microscopic evaluation of *ex vivo* cells demonstrated substantive restoration of actin accumulation at and lytic granule polarization to the IS.⁸ Interestingly, the quantitative analyses demonstrated 2 distinct populations of NK cells: one restored and one not. The more recent study of lentiviral gene therapy identified that approximately 50% of NK cells were gene corrected, and direct quantitative comparison by means of confocal microscopy was used to measure the ISs of WASp⁺ and WASp⁻ NK cells within the same patient. These analyses showed restoration of F-actin accumulation and granule polarization in those lymphocytes expressing WASp but not in those that did not have restored WASp expression.⁹ In addition, NK cell cytotoxic function was restored in these patients (along with platelet counts and T-cell proliferation), and they all had documented clinical improvements, including protection from bleeding and resolved eczema. Despite the well-deserved caution with which gene therapy is approached, in this case the lentiviral vector approach has resulted in successes, with no patients showing evidence of clonal expansion after 3 years (unlike with retroviral gene therapy).

After the discovery that WAS is linked to cytoskeletal abnormalities, microscopy technology has been applied to the biology of other cell types in patients with WAS, particularly platelets and megakaryocytes, because small platelet size and thrombocytopenia are consistent hallmarks of WAS and X-linked thrombocytopenia. There are multiple actin-dependent processes involved in proplatelet formation, including their generation from megakaryocytes and the transendothelial migration required to release platelets into the circulation. Platelets are produced from bone marrow megakaryocytes, which extend long processes known as pseudopodia that subsequently break off at constriction sites to form proplatelets.⁴⁹

An early study described seemingly normal platelet-producing pseudopodia in CD34⁺-derived megakaryocytes from both patients with WAS and healthy donors when viewed by using light microscopy, an observation that was surprising given the importance of the cytoskeleton in the generation of pseudopodia.¹⁰ Closer examination identified aberrant F-actin accumulation in megakaryocytes from patients with WAS through fluorescence imaging. Specifically, an atypical

accumulation of F-actin was demonstrated in the center of cells along with a paucity of cortical actin. This was further confirmed by means of electron microscopy of the fine structure of F-actin in normal and WAS platelet-producing megakaryocytes. Importantly, these observations demonstrated that platelet diminution occurs in the periphery after development and collectively underscore the complexity of F-actin regulation in megakaryocytes. The mechanism of abnormal platelet homeostasis in patients with WAS remains poorly understood; hopefully, recent advances in imaging applied to patients' cells will provide further insights.³⁹

Finally, an intriguing role for WASp as a nuclear regulator in the context of WAS was described in part by using fluorescence microscopy. This work demonstrated that WASp localizes to the nucleus and plays a role in the control of transcriptional regulation of the *TBX21* transcription factor.¹¹ The process was abnormal in T cells from patients with WAS and can potentially contribute to some of the T-cell deficiency found in patients with this disease. Although fluorescence or confocal microscopy has been the norm for such experiments evaluating nuclear localization, the advent of high-throughput technology, such as imaging flow cytometers, will likely increase access to informative localization data for other proteins involved in WAS and other complex immune deficiencies regarding transcription factor regulation and nuclear protein access.

Recently, PIDs affecting other actin regulators have been identified. These extend the contributions that have been made to our understanding of cell biology through this category of primary immunodeficiencies facilitated by quantitative imaging. These newer diseases include both regulators of actin nucleation and deconstruction. Dedicator of cytokinesis 8 (DOCK8) is a guanine nucleotide exchange factor that activates CDC42 and thus WASp. DOCK8 deficiency was identified as a cause of autosomal recessive hyper-IgE syndrome.⁵⁰ Patients with DOCK8 deficiency are affected by sinopulmonary and cutaneous infections and severe allergies. In addition, many have recalcitrant herpes and papillomavirus infections, which are classical hallmarks of NK cell deficiency.⁵¹ Accordingly, both NK cells from DOCK8-deficient patients and DOCK8-deficient NK cell lines have deficient NK cell cytotoxic function. In contrast to NK cells from WAS-deficient patients, DOCK8-deficient NK cells have normal baseline F-actin levels.¹² However, CDC42 is specifically invoked in response to activation, and therefore there is a defect in F-actin accumulation at the synapse of NK cells from DOCK8 patients in conjugation with susceptible target cells, as measured quantitatively by using confocal microscopy (Fig 1, B).¹² This is accompanied by a defect in the accumulation of integrins at the synapse and lytic granule polarization, which are both known to be actin-dependent processes.^{4,13} Granule convergence to the MTOC, which occurs before both these steps and was previously reported to be independent of F-actin regulation,⁵² was conserved. Perhaps more interestingly, unlike in the context of WAS, IL-2 stimulation did not restore NK cell function or F-actin accumulation at the lytic synapse (either as short-term stimuli or in the context of bulk culture). This suggests that DOCK8 might be a more general upstream regulator of actin activators, such as WASp and WAVE2, or that there might be branched pathways of actin activation. This mechanism was further dissected by biochemical studies showing that DOCK8 directly interacts with the cytoskeletal linker talin and WASp and is required for their recruitment to the synapse.⁵³

The strength of novel imaging technology, as well as the complexity of actin regulation at the immune synapse, was recently highlighted by the identification of a mutation in Coronin 1A as a rare cause of PID. Coronin 1A promotes F-actin turnover through both the regulation of cofilin (which severs F-actin filaments) and the negative regulation of the Arp2/3 complex (the actin branch facilitator). Previous immunologic defects associated with Coronin 1A mutation in both mice and human subjects include impaired T-cell migration and thymic egress, likely caused by misregulation of F-actin.^{54,55} The latter was demonstrated by means of confocal microscopy in a mouse model of the disease but not in patients' cells because of an initial scarcity of samples.

A further insight into the effect of Coronin 1A mutation was determined through application of superresolution microscopy to the investigation of fine F-actin structure and was observed in patients' cells directly. This revealed a critical role in the deconstruction of F-actin as opposed to the generation of branched F-actin networks. This work demonstrated that the focal and targeted deconstruction of F-actin is required for the access of lytic granules to the plasma membrane in NK cells.¹⁴ This corresponds with a defect in NK cell cytotoxic function and likely contributes to patient disease. Specifically, there is a preponderance of herpesvirus infections in patients with Coronin 1A deficiency.^{54,56,57} Importantly, discovery of the fine actin structure defect in patients with Coronin 1A deficiency was only enabled by the use of superresolution imaging because the defect was not detected by means of conventional confocal imaging. As such, the discovery represents the first published application of superresolution imaging in immune cells from patients with PIDs.

The importance of Coronin 1A in F-actin deconstruction for lytic granule access to the plasma membrane complemented earlier work describing the importance of nonmuscle myosin IIA in NK cell cytotoxicity. The myosin superfamily, members of which generate ATP-dependent movement along actin filaments, play numerous roles in adhesion, migration, and immune function.⁵⁸ Mutations in the myosin II heavy chain lead to MYH9-related disease, which is characterized by hematologic defects, including macrothrombocytopenia and in some cases cataracts, deafness, and glomerulonephritis.⁵⁹ Through use of confocal microscopy, TEM, and total internal reflection fluorescence microscopy, a critical role for myosin IIA in NK cell cytotoxicity was revealed, and a previously unappreciated immune cell defect in MYH9-related disease was defined. Myosin IIA had previously been reported to accumulate at the NK cell lytic synapse, and treatment with myosin IIA inhibitors abrogates NK cell cytotoxic function.⁶⁰ By using biochemical, proteomic, TEM, and microscopic approaches, it was shown that myosin IIA is constitutively associated with NK cell lytic granules. These findings were furthered by the definition of phosphorylation of the myosin tailpiece as being required for its interaction with granules.¹⁵ In what was the first application of superresolution imaging to NK cells, individual myosin molecules were visualized and quantified on NK cell lytic granules, providing striking evidence of the link between granules, F-actin, and myosin. Although not quantified in these studies, as in Coronin 1A mutated cells, lytic granules appear unable to penetrate synaptic actin in the absence of myosin function (Fig 1, B), suggesting that myosin might be required for generating the force required to mobilize relatively large lytic granules across synaptic actin.^{15,16,61}

Collectively, these studies combining quantitative microscopy and human immune deficiency have contributed significantly to our understanding of the regulation and function of the immune cell cytoskeleton. One of the powerful aspects of using quantitative microscopy is the ability to extract highly resolved data when experimental conditions are carefully controlled and thoughtfully executed. This often occurs through the creative application of measurement algorithms to rigorously study the question at hand. As imaging technology continues to innovate, it is imperative that physician-scientists and cell biologists continually approach the data with the goal of extracting maximal information in an objective manner. This includes collaboration with computational biologists and mathematicians, who can offer unbiased and novel approaches to biological questions.

CELL SIGNALING DEFECTS

Immune cell effector function is a critical component of both the innate and adaptive immune responses. In both cases signaling is tightly regulated and delicately balanced, resulting in fine control of directed secretion and other effector functions. In addition to those PIDs affecting cytoskeletal processes, there are an increasing number being identified that affect signaling, leading to IS formation and function. Many of the intricacies of these diseases have been resolved by using quantitative imaging.

Integrins bridge the actin cytoskeleton and cell-signaling molecules and are critical for immune cell function. In particular, lymphocyte function-associated antigen 1 (LFA-1) plays an indispensable role in T-cell adhesion, migration, extravasation, and scanning of dendritic cells (DCs) in lymph nodes.⁶² LFA-1 provides the crucial adhesive force and actin rearrangement required for IS formation in T and NK cells. It also directs secretion of lytic granules in cytotoxicity and is required for their convergence to the MTOC in NK cells.⁵² LFA-1 function is tightly regulated by its conformational state and can be rapidly activated by signaling that increases affinity for its primary ligand, intercellular adhesion molecule 1. In addition, after ligand binding, LFA-1 undergoes conformational activation and also mediates signaling itself, leading to the dynamic actin rearrangement that is required for cytotoxic function. Finally, LFA-1 plays a critical role in the migration of neutrophils and platelet adhesion. Patients with leukocyte adhesion deficiency type 1 (LAD-I) exhibit decreased cytolytic effector function, which was thought to be due to reduced adhesion to targets; however, at least in NK cells, the scenario appears to be more complex.

Microscopy studies of cells from patients with LAD-I are limited to the biology of NK cells, in which LFA-1 functions as an early signal for lytic granule convergence to the MTOC before MTOC reorientation and directed secretion of granules.⁵² Using fixed cell confocal microscopy, it was shown that NK cells from patients with LAD-I do not converge lytic granules to the MTOC after conjugation with susceptible target cells.¹⁷ Because this initial convergence is a critical step in the subsequent directed lytic granule secretion for NK cell cytotoxicity, this observation likely accounts for some of the severe defects demonstrated by NK cells from patients in cytotoxicity assays. Adhesion to target cells, although decreased at early time points, was not significantly impaired by 60 minutes in cell from patients with LAD-I. This somewhat surprising finding raises the possibility

of alternate ligands functioning in firm adhesion in NK cell–target conjugates; however, the absolute lack of cytotoxic function in NK cells from patients with LAD-I underscores the importance of LFA-1 in NK cell function, as well as the insights gained from quantitative imaging.

An interesting new class of PID has recently been identified that defines the importance of divalent cation signaling in effector function. Although Ca^{2+} is a well-documented second messenger, roles for Mg^{2+} and Zn^{2+} as important signaling molecules are now also known.⁶³ The importance of cation signaling is underscored by the discovery of a PID that occurs as a result of mutations in Mg^{2+} or Ca^{2+} channels. In the case of Mg^{2+} signaling, mutations in the magnesium transporter 1 (*MAGT1*) gene result in X-linked immunodeficiency with Mg^{2+} defect, EBV infection, and neoplasia disease.¹⁸ Genetic deficiency of *MAGT1* leads to defects in TCR-mediated T-cell activation and CTL effector function. Interestingly, it also impairs NK cell function in part because of decreased cell-surface expression of the important activating receptor NKG2D, levels of which were also decreased on T cells and even EBV-transformed B-cell lines from patients. This was demonstrated in part by using quantitative confocal microscopy. The presence of exogenous Mg^{2+} supplemented either in cell medium or, impressively, as an oral therapy restored NKG2D levels on patients' cells, as well as effector cell function.¹⁸ The signaling leading from the TCR (or NK cell activating receptor) to *MAGT1* is poorly understood, and no doubt the application of molecular biology and quantitative imaging to this question will yield further novel understanding of these signaling pathways.

The importance of Ca^{2+} and the signaling pathways leading to Ca^{2+} channel regulation are better understood. Store-operated calcium entry is required for T-cell and NK cell activation, sustained signaling, and exocytosis of lytic granules.⁶³ Mutations in *ORAI1* or stromal interaction molecule 1 (*STIM1*), both of which are required for store-operated calcium entry in NK and T cells, result in impaired NK cell degranulation and cytotoxicity.¹⁹ In NK cells this effect seems to be primarily on the relatively late degranulation step because confocal microscopy of NK cells from *ORAI1*-deficient patients showed normal granule convergence and polarization.¹⁹

The nuclear factor κB (NF- κB) pathways are critical components of the innate immune system. The canonical pathway is activated in response to signaling receptors and results in formation of a signalosome and assembly of the IKK complex, which contains IKK α , IKK β , and IKK γ (NEMO). Canonical signaling ultimately leads to p65 translocation to the nucleus. Less commonly, activation through CD40/B cell-activating factor of the TNF family receptor/lymphotoxin β receptor leads to noncanonical pathway activation, as well as the NF- κB -inducing kinase (NIK)-dependent, NEMO-independent processing of p100 to p52 and its subsequent nuclear translocation. Although not commonly associated with NK cell activation signaling, the canonical NF- κB pathway is rapidly induced after ligation of the NK cell activating receptor NKP30.²⁰ This likely and at least partially accounts for the defect in NK cell cytotoxicity in patients with IKK/NEMO mutations.⁶⁴ Confocal microscopy shows rapid translocation of p65, but not p50 or p52, to the nucleus when freshly isolated peripheral NK cells were conjugated to susceptible K562 targets.²⁰ Recently, loss-of-function mutations in NIK leading to novel PIDs were

described.²¹ Loss of NIK led to defects in both canonical and noncanonical NF- κB signaling and included defects in B-cell class-switching and T-cell responses. There was also impaired NK cell function in patients with NIK deficiency, which was analyzed by using confocal microscopy and described a failure to accumulate F-actin at the synapse and polarize lytic granules. NK cells were present at a decreased frequency in peripheral blood of patients when compared with that of healthy control subjects and were defective in cytokine secretion. Whether this is a feature of decreased NK cell survival, impaired terminal maturation, or abnormal homeostasis is presently unclear.

Although inroads into understanding immune cell signaling in patients with PIDs by using advanced microscopy have led to a number of important advances, the temporal nature of signal generation will benefit from high-resolution and superresolution studies in living cells. The newer microscopy technologies available as applied to living cells should allow for acceleration of scientific progress in patients with this particular subset of disorders.

INTRACELLULAR TRAFFICKING DEFECTS

Modern microscopy techniques applied to PIDs have had perhaps the greatest effect on the understanding of defects in cytotoxicity mediated by both T and NK cells. This immune effector function is dependent on dual-function secretory lysosomes that function as organelles specialized both for lysosomal function and secretion of perforin and granzymes (Fig 2, A). This specialized secretory lysosome function is also used in pigment-producing melanocytes through the melanosome, and many components of the secretory machinery are shared between melanocytes and cytolytic immune cells. For this reason, mutations leading to defects in secretory lysosome formation and function also can result in aberrant pigmentation of affected patients. The molecular biology of diseases caused by perturbation of lytic granule contents, biogenesis, docking, and secretion have been comprehensively reviewed elsewhere.^{41,51,65,66} Frequently, mutations in these pathways cause hemophagocytic lymphohistiocytosis because of abrogation of cytotoxicity without impairment of proinflammatory function.

The use of quantitative imaging has proved essential in elucidating both the biology and disease-causing aspects of genetic mutations leading to Griscelli syndrome 2 (Rab27a), Hermansky-Pudlak syndrome (adaptor protein 3 [AP3]), familial hemophagocytic lymphohistiocytosis (FHL; perforin, Munc13-2, Munc18-4, syntaxin 11), immune deficiency as a result of p14 mutation (p14), and Chediak-Higashi syndrome (CHS; lysosomal trafficking regulator [*LYST*] gene; Fig 2, B).^{22-28,31,32}

Albinism and giant lytic granules in immune effector cells accompany CHS, Griscelli, and Hermansky Pudlak syndrome type 2 (HPS2) syndromes because all diseases affect the common granule-trafficking pathway shared between immune effectors and melanocytes. Before the identification of mutations in *LYST* being causative for CHS and the *beige* mutation in mice, the early observation that giant lysosomes accumulated in CTLs and did not undergo normal exocytosis was made by using confocal microscopy.²² This led to the conclusion that the immune defect in patients with CHS at least partially arose from loss of secretion in CTLs and raised the hypothesis that the protein involved played a role in vesicle membrane fusion or exocytosis.²² Interestingly,

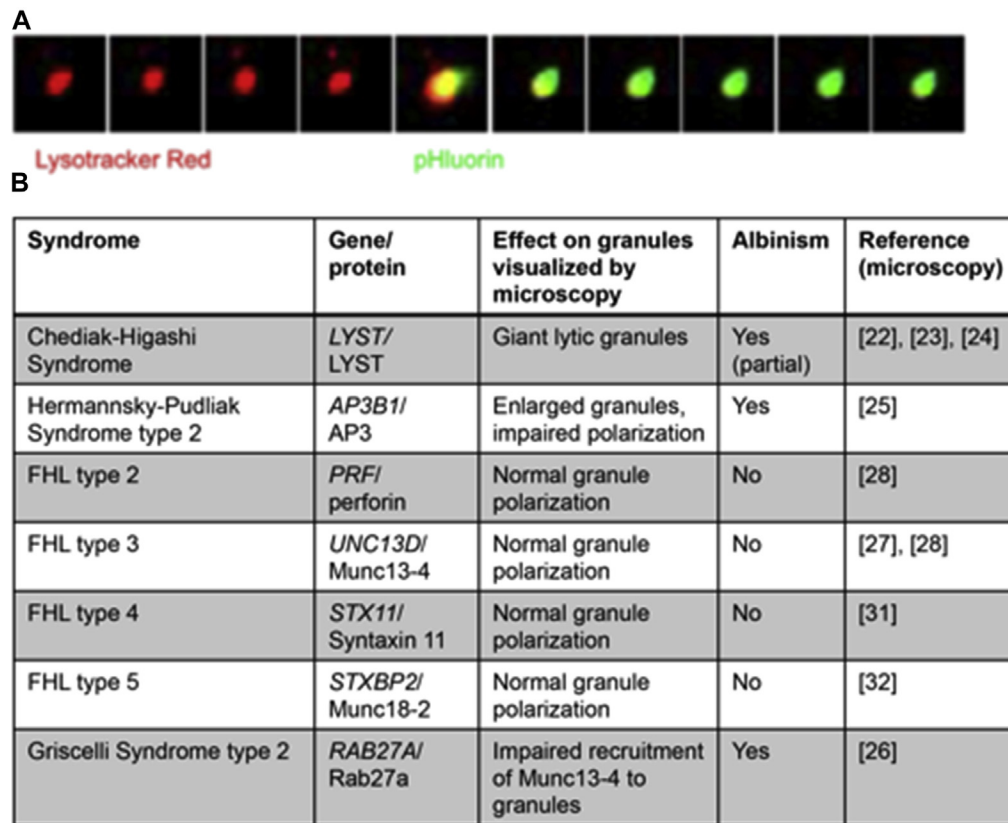


FIG 2. Intracellular trafficking leads to lytic granule exocytosis. **A**, Lytic granules from NK cells expressing the degranulation indicator LAMP1-pHluorin were labeled with LysoTracker Red for visualization before degranulation. Shown is a single granule undergoing exocytosis as marked by a transition from acidified organelle (red) to neutralization and exposure of the pH-sensitive green fluorescent protein variant to the neutral extracellular environment. The image was acquired by means of total internal reflection microscopy at the plasma membrane of an NK cell activated on glass. **B**, Table of PIDs affecting intracellular trafficking and their effect on hair pigmentation and lytic granule polarization. Microscopy references refer to those cited in this review that have examined the specific effect on granule behavior through quantitative microscopy.

lysosomes are formed normally in patients with CHS and then increase in size because of fusion, or abnormal fission, of lytic granules, as shown by using electron microscopy (in naive *in vitro* activated CTL clones from patients with CHS).^{22,23} Ultimately, this process results in 1 to 3 giant lysosomes per cell.^{22,23} Further biological insight was gained when the gene for CHS was identified, and molecular studies identified *LYST* as being required for the regulation of lysosomal trafficking,⁶⁷⁻⁶⁹ which is now more clearly ascribed to the generation of individual secretory lysosomes and, in particular, the terminal maturation of perforin-containing granules into exocytosis competent granules.²⁴ These elegant new studies performed in part with total internal reflection microscopy, confocal microscopy, and correlative light electron microscopy show that enlarged granules result from the fusion of normal-sized endolysosomal organelles and, interestingly, that even those lytic granules that fall within the normal size range have impaired motility and ability to degranulate at the synapse.²⁴ Finally, the overexpression of effectors involved in degranulation was able to restore the ability of CTLs from patients with CHS to mediate function, thus underlining the role that *LYST* is playing in the trafficking of effectors to the key sites of action.

HPS2, which also affects the late stages of granule biogenesis, is caused by mutation of the *AP3B1* gene, which encodes the AP3 protein. AP3 is required for sorting of granule components from the Golgi into lysosomes. Granzyme and perforin are appropriately localized in lytic granules from CTLs from patients with HPS2 based on confocal microscopy studies of granule components.²⁵ Specifically, CTL clones derived from patients with HPS2 were stained with antibodies to lytic granule components and showed strong colocalization of both lysosome-associated membrane protein 2 and perforin with cathepsin D, indicating appropriate localization of lytic granule constituents. Interestingly, the well-defined defect in CTL cytotoxicity in patients with HPS2 was described by using confocal microscopy to be due to impaired transit of lytic granules along microtubules, resulting in defective polarized secretion. When examined by using TEM, granules from patients with HPS2 are also enlarged and contained many more multivesicular structures in comparison with those from healthy donors, which have an electron-dense core on maturation.^{23,25,70} NK cell cytotoxicity is also impaired in patients with HPS2, and although NK cells from patients with HPS2 have not been specifically described by using microscopy, it is thought that AP3 is playing

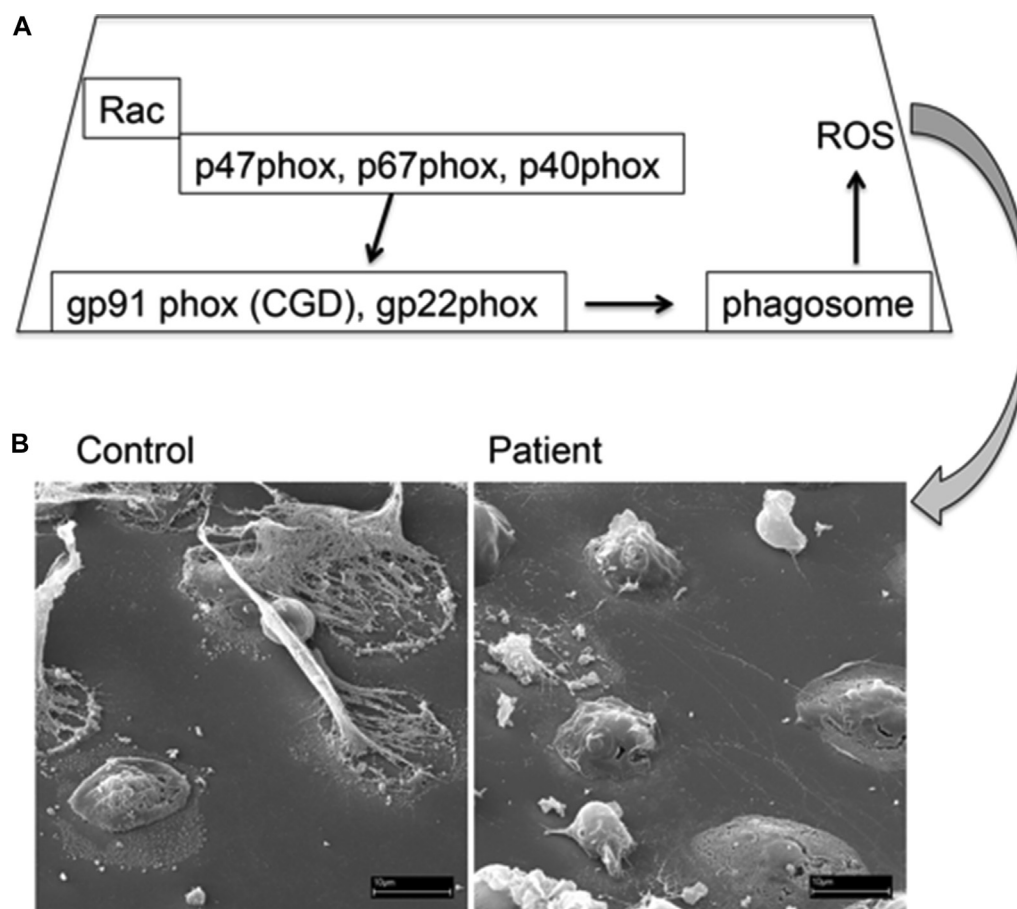


FIG 3. Cellular host defense through formation of NETs. **A**, NET formation requires both phagosome formation and ROS. **B**, NET formation is impaired in neutrophils from patients with CGD. Neutrophils from control subjects (*left*) or patients (*right*) were stimulated with phorbol 12-myristate 13-acetate and ionomycin and imaged 5 hours later by using scanning electron microscopy. Scale bar = 10 μ m. Image courtesy of Dr Janine Reichenbach, University Children's Hospital Zurich.

a similar role in NK cells.⁷¹ Although the exact mechanism of this is unknown, it might be that a second protein required for lytic granule attachment to or movement on microtubules is inappropriately localized in the absence of AP3.

Although Griscelli syndrome 2 is also associated with albinism, the Rab27a mutation results in normal granule polarization but a defect in lytic granule docking and subsequent degranulation based on confocal microscopy studies done in mouse CTLs and Rab27a-deficient NK cell lines.⁷²⁻⁷⁵ In NK cells ligation of activating receptor recruits both Rab27a and Munc13-4 to lytic granules, which was shown by measuring their colocalization with perforin in confocal microscopy images.²⁶ NK cells from a patient with Griscelli syndrome 2 were impaired in their ability to recruit Munc13-4 to granules, suggesting that Rab27a is required for assembling the secretory lysosome docking complex.²⁶

UNC13D, which encodes Munc13-4, is mutated in patients with FHL type 3. Both NK cells and CTLs have defective cytotoxic function in these patients, despite demonstrating normal lytic granule polarization toward target cells, as visualized by using confocal microscopy and TEM.^{27,28} This suggests a defect in the exocytosis of granule contents at the plasma membrane, a poorly understood step of cytotoxicity. Exocytosis

includes docking and priming steps mediated by proteins, such as Munc18-2, Rab27a, and Munc13-4. This is followed by membrane fusion mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), such as syntaxin 11. Rab27a and Munc13-4 are recruited to lytic granules independently, as demonstrated through confocal microscopy, which suggests that the role of Munc13-4 might also be to recruit and prime Rab27a.^{29,30} In addition, the structure and function of Munc13-4 suggest that it serves a role in SNARE priming. The importance of SNAREs in NK cell cytotoxicity is underscored by the absence of NK cell degranulation in patients with FHL type 4, which is caused by mutations in the *STX11* gene.²⁷ Similar to the previously described docking and priming defects, syntaxin 11-deficient NK cells have normal granule polarization toward the target cell, as demonstrated by using confocal microscopy of cells from patients with FHL4.³¹ Munc18-2, which is encoded by the *STXBP2* gene, is mutated in patients with FHL type 5. Munc18-2 colocalizes with syntaxin 11, as measured in quantitative imaging experiments. Confocal microscopy with NK cells from patients with FHL type 5 again shows normal lytic granule polarization but defective degranulation.³² Together, these demonstrate the biological and clinical relevance of the secretory lysosome docking and fusion complexes, the

understanding of which would be minimal were it not for the application of advanced quantitative imaging approaches.

More recently, a novel role for the adaptor in p14 human disease was defined as causing a PID with partial albinism, short stature, congenital neutropenia, and B- and T-cell lymphopenia and a form of hemophagocytic lymphohistiocytosis.³³ Further studies defined by using quantitative confocal microscopy identified defects in lytic granule biogenesis. TEM of keratinocytes and immune cells showed abnormal melanosome distribution in keratinocytes and debris-containing phagosomes in the neutrophils of patients; however, the characteristic inclusion bodies seen in neutrophils of patients with CHS were not observed. Reconstitution of the defect in fibroblasts and subsequent confocal microscopy showed that late endosomes were not targeted to the perinuclear region, as they were in control cells. Loss of CD8⁺ cytotoxic function was observed, but defects in granule release were not, which suggests some more inherent abnormality in the granules themselves in patients with p14 deficiency. The application of quantitative confocal microscopy to this novel condition has enabled the definition of a novel signaling pathway and a previously unappreciated role for p14 in immune cell function.

DEFECTS IN CELLULAR HOST DEFENSE

Light and electron microscopy have been used to richly define genetic disorders affecting cellular host defenses against pathogens provided by neutrophils and macrophages. In particular, this includes those affecting a visually striking and critical role of neutrophils: formation of neutrophil extracellular traps (NETs). NETs are extruded lattices of DNA, histones, granule enzymes chromatin, and extracellular proteins that enable the destruction of microbes, fungus, and even viruses by neutrophils.⁷⁶⁻⁷⁸ The discovery of NETs would not have been possible were it not for extraordinary attention to detail using high-resolution imaging because these delicate and enigmatic structures were initially very difficult to characterize by using lower-resolution methods. NET generation requires the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and as such, their formation is impaired in patients with chronic granulomatous disease (CGD), as well as in patients with acquired neutrophil dysfunction disorders and hereditary disorders of unknown cause.

The initial description of neutrophil NETs resulted from a microscopy-driven discovery based on the surprising observation that activated neutrophils produced fragile yet visible extracellular projections when visualized by means of both electron and immunofluorescence microscopy.⁷⁶ Immunostaining and TEM identified histones and neutrophil elastase as NET components and provided striking images of both gram-negative and gram-positive bacteria trapped in NETs. These data were supported by *in vitro* assays showing decreased bactericidal activity of neutrophils after DNase treatment and *in situ* immunohistochemistry showing the presence of NETs in tissues. Generation of NETs is dependent on generation of reactive oxygen species (ROS), after which neutrophil elastase and myeloperoxidase trigger the decondensation of chromatin and the subsequent release of NETs into the extracellular space (Fig 3, A). Because of this dependence on ROS, NET formation in patients with CGD is also impaired (Fig 3, B).³⁴ This was defined by using confocal microscopy of neutrophils isolated

from patients with CGD. Interestingly, neutrophils from patients with CGD retain the ability to form NETs when stimulated with glucose oxidase³⁴; however, after infection with *Staphylococcus aureus*, NET formation was impaired. Both these clinically relevant observations were made by using immunostaining of neutrophils isolated from patients with CGD.

In patients with CGD, mutations in any one of 5 NADPH oxidase subunits (membrane-bound gp91^{phox} and p22^{phox} and cytosolic p47^{phox}, p67^{phox}, and p40^{phox}) can impair ROS generation, leaving patients highly susceptible to refractory bacterial and fungal infections. Activation of NADPH oxidase by ingestion of bacteria or fungi generates phagosome superoxide, hydrogen peroxide, and hypochlorous acid, followed by rapid influx of potassium ions to the phagosome, which activates antimicrobial peptides.^{79,80} Activation of the pathway results in binding of the cytoplasmic components to each other, followed by their translocation with the GTPase Rac to the plasma membrane and association with membrane-bound components and, likely, the actin cytoskeleton.⁸¹⁻⁸⁵ The subcellular localization of these components was intricately defined by the combination of microscopy with biochemical phagocytosis assays to detect bacterial opsonization.^{35,86,87} Loss of gp91^{phox} most likely affects complex formation through the inability of the cytosolic complex to dock at the plasma membrane, and studies with p47^{phox}-deficient subjects show that p67 recruitment requires p47^{phox}.³⁵ p47^{phox} and p67^{phox} are recruited normally to phagosomes in PMNs from both healthy subjects and patients with CGD but are shed with the cytoskeletal proteins, as opposed to normal PMNs, in which p47^{phox} and p67^{phox} are retained with the forming phagosome. This was shown by using fixed cell fluorescence microscopy of adherent neutrophils opsonizing bacteria. After fixation, cells were stained with antibody to the NADPH oxidase pathway components of interest, p57 or phalloidin, to detect F-actin. The images clearly demonstrate a rapid colocalization of F-actin and p57, which is lost at later time points. In addition, they show retention of p47 and p67 at later time points in PMNs from healthy subjects but not patients with CGD. Relatively straightforward scoring of positive versus negative fluorescent intensity within phagosomes provided quantitative data to support the conclusions seen by eye. This study highlighted the importance of the cytoskeleton and its regulation in the NADPH oxidase pathway through use of microscopy.

Gene therapy to restore gp91^{phox} expression might be an option for treatment in certain patients with CGD because expression of a monocistronic long terminal repeat-driven gamma-retroviral SF71-gp91^{phox} vector in a boy with X-linked CGD resulted in a visible increase in NET formation and clearance of *Aspergillus* species hyphae after *in vitro* infection of the patient's neutrophils with *Aspergillus* species isolated from his own disease.³⁶ This shows the importance of NET formation in the control of hyphae, which are likely too large to phagocytose. A further therapeutic pathway explored *in vitro* in cells from patients with CGD is the use of liposomes to deliver glucose oxidase.³⁷ Confocal microscopy and TEM show that the uptake of bacteria and liposome-derived particles and treatment with glucose-oxidase containing liposomes increased the killing of *S aureus* by granulocytes from patients with CGD.

Finally, in a mouse model of CGD, the importance of CD4⁺ T cells in the control of *Aspergillus* species infection was illustrated by using confocal microscopy, underscoring the role of the

NADPH pathway in DC cross-priming of T cells.⁸⁸ This clearly demarcated the intracellular pathway of processing of *Aspergillus* species conidia by means of confocal staining for lysosomal pathway components and fluorescently labeled fungal antigen. This showed impaired lysosomal localization in DCs from patients with CGD and the resulting impaired T-cell response. Thus through an advanced microscopy-based subcellular localization study in patients with PIDs, mechanistic insight was gained into a perplexing abnormality present in the T cells of patients with CGD.

CONCLUSION

An increase in the technology available for rapid and accurate diagnosis of PID has resulted in a correlative increase in the opportunity for important cell biology discoveries that can be made in parallel. With the advent of relatively accessible whole-exome sequencing, chromosomal array, and bioinformatics analyses to support these technologies, the genetic cause for disease in patients with rare PIDs is becoming more frequently known early after diagnosis. Equally brisk progress in cell biology and the improved microscopy tools available to probe the inner secrets of immune cells has paralleled these advances. Applying the knowledge of genetic perturbation to the immunologic and cell biological phenotype in afflicted subjects gives us the power to unravel cell-signaling pathways and intracellular mechanisms, leading to increased understanding of the molecular basis of disease. In concert, these advances will undoubtedly accelerate progress toward the common goal of therapeutic advances in the treatment of these unique patients.

We thank all patients with PIDs for their contribution to science, because without them this research would not be possible. We also thank our clinical and research colleagues for ongoing collaboration and discussion of topics included in this review. We especially thank Dr Janine Reichenbach for the contribution of the electron micrographs for Fig 3.

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