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Insights into Primary Immune Deficiency from quantitative microscopy analysis

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Abstract

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.

Keywords

Primary Immune Deficiency; microscopy; cell biology; cytoskeleton; host defence

Introduction

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 immunological 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 diagnosis and treatment of these often rare, devastating and complex diseases.

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In parallel with genetic technology, the field of optical imaging has undergone a rapid revolution in the past ten years through the 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 have enabled improvements in molecular localization, optical sectioning, 3-dimensional reconstruction, live cell imaging and even 4-dimensional reconstructions (Table 1). One of these technical advances, super-resolution imaging, is particularly noteworthy as it has dramatically decreased the size of resolvable objects within cells from previous limits of about 200nm to new reaches of even single digit nm levels through the use of optics. Recognition for this powerful advance was provided via the 2014 Nobel Prize in Chemistry and Physics.

The application of highly quantitative imaging, objective image analysis and, more recently, super-resolution 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 human disorders, often in children from whom samples can be scarce.

Here, we review the application of highly quantitative imaging to the field of PID with a specific focus on three areas that have particularly benefited from this increased resolution, namely those 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 filamentous- (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 [1]. As such, mutations affecting the cytoskeleton have a profound affect and lead to significant disease [1]. In particular, the regulated control of F-actin nucleation and remodeling is required for the formation and function of the immunological synapse (IS) (Figure 1A). The IS can be broadly described as a highly specialized signaling platform formed between two 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, may be the so-called “lytic synapse” that leads to the directed secretion of specialized secretory lysosomes, or lytic granules. Both cytotoxic T lymphocytes (CTL) and natural killer (NK) 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 numbers of discrete steps [2]. Central to an increasingly appreciated number of these is the highly regulated function of the cytoskeleton (Table 2), 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, has been well reviewed elsewhere [1].

The study of PIDs affecting the regulation of F-actin have led to critical insight into 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 WASp have been described leading to classical WAS, X-linked thrombocytopenia or X-linked neutropenia [3]. WAS as a heritable condition was first described in 1954 and is recognized clinically by the characteristic triad of thrombocytopenia, neutropenia and immune deficiency [3]. 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 [4]. WASp (and its homologues) activates the 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. This provides tremendous structural integrity akin to interlocking girders in a building. While 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 [3].

WAS was one of the first PID to be studied from a cell-biological perspective using higher resolution microscopy. Scanning electron microscopy was initially utilized to study lymphocytes from WAS patients [5] and semi-quantitatively score the presence and relative size of microvillus projections on the cell surface. The morphological abnormalities observed in patient 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 [6, 7] [8] via electron microscopy with regards 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 [5-7] [8]. Further, the effects seen in WAS patient cells can be variable, including some “baldness” but also altered and heterogeneous morphology, especially in T cell lines. B cells from WASp-deficient mice showed some microvillus defects [8], but these experiments also utilized in vitro cellular activation. The cell surface morphology of WAS patient NK cells has never been specifically examined by electron microscopy. In light of the divergent response of NK cells from T cells in regards to IL-2-induced actin polymerization (see below), it would be of interest to determine if WAS NK cells have more profound microvilli defects at baseline.

Cloning of the WAS gene [4] led to identification of Cdc42 as the target of WASp control [9] 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, however, that demonstrated that the defect in the formation and

function of the actin-directed immunological synapse is what leads to loss of NK cell effector function in WAS patients. Loss of WASp function leads to impaired F-actin accumulation at the NK cell IS and accompanying loss of perforin polarization [10]. 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 regards 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 confocal microscopy. Also in contrast to WASp-deficient NK cells, CTLs degranulate at a frequency comparable to cells from healthy donors [11].

While no difference in frequency of conjugate formation between WAS CTL 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, naïve CD4+ T cells from WASp^{-/-} mice have been shown to be incapable of stable synapse formation after migration [11, 12]. Interestingly, the CD4+ T cell synapse from cell lines derived from WAS patients showed abnormal F-actin protrusions and morphology, but demonstrated normal conjugation efficiency with APCs. Three-dimensional reconstruction confocal microscopy showed a decrease in the surface area of WAS CD4+ T cell synapses formed with APCs. As in NK cells, there was also a lack of MTOC polarization to the synapse. This abnormality in WAS T cells 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, as it demonstrated that initial T cell receptor accumulation and phosphorylation of tyrosine residues at the synapse was normal but then de-stabilized over time. For the first time, this suggested an inability for WAS T cells to maintain TCRs clustered at the synapse. In addition, the kinetic imaging showed that while 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 WAS CTL may 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 WAS patient cells [10, 13] [14]. This was initially defined as a macroscopic observation in ⁵¹Cr-release functional assays and was 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 [13]. This novel WASp-independent signaling pathway downstream of IL-2 was identified 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 as compared to T cells, in which WAVE2 is accessed constitutively for normal F-actin function [15]. 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 (Figure 1B). While 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 architecture of actin filaments are easily discerned and even measured.

The new insights into IL-2 in inducing WAVE2 function in WAS patient NK cells has led to a promising and presently active clinical trial to study the application subcutaneous IL-2 injections in WAS patients (NCT00774358). The rationale for IL-2 application in WAS is that there could be benefit for restoration of NK cell function given the susceptibility of patients to herpesvirus infections and hematological malignancies. The use of confocal microscopy to quantify the cytotoxic function of patient NK cells following in vivo administration of IL-2 has provided some early support to the biochemical data in that there was a significant increase in the basal levels of F-actin in patient NK cells [13], as well as restoration of polarization of the cytolytic machinery towards the target cell.

A second novel approach for the treatment of WAS is through the use of gene therapy. Autologous CD34+ hematopoietic stem cells were transduced with full length WAS gene initially via the use of a retroviral vector [16] and subsequently a lentiviral vector utilizing the endogenous WASP promoter [17]. In the earlier work, confocal microscopic evaluation of ex vivo cells demonstrated substantive restoration of actin accumulation at, and lytic granule polarization to, the immunological synapse [16]. Interestingly the quantitative analyses demonstrated two 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 confocal microscopy was used to measure the immunological synapses 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 [17]. 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 the retroviral gene therapy).

Following the discovery that WAS is linked to cytoskeletal abnormalities, microscopy technology has been applied to the biology of other cell types in WAS patients; particularly platelets and megakaryocytes, as small platelet size and thrombocytopenia are consistent hallmarks of WAS and XLT. There are multiple actin-dependent processes involved in pro-platelet 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, which subsequently break off at constriction sites to form proplatelets [18]. An early study described seemingly normal platelet-producing pseudopodia in both WAS and healthy donor CD34+-derived megakaryocytes when viewed by light microscopy, an observation that was surprising given the importance of the cytoskeleton in the generation of pseudopodia [19].

Closer examination did identify aberrant F-actin accumulation in WAS megakaryocytes via 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 electron microscopy of the fine structure of F-actin in normal and WAS platelet-producing megakaryocytes. These observations demonstrated, importantly, that platelet diminution occurs in the periphery following development and collectively underscore the complexity of F-actin regulation in megakaryocytes. The mechanism of abnormal platelet homeostasis in WAS patients remains poorly understood and hopefully recent advances in imaging applied to patient cells will provide further insights [1].

Finally, an intriguing role for WASp as nuclear regulator in the context of WAS was described in part by 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 (T-bet) [20]. The process was abnormal in T cells from WAS patients and can potentially contribute to some of the T cell deficiency found in the disease. While currently 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, primary immune deficiencies 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 [21]. Patients with DOCK8 deficiency are affected by sinopulmonary infection, cutaneous infections and severe allergies. In addition, many have recalcitrant herpes and papillomavirus infections, which are classical hallmarks of NK cell deficiency [22]. Accordingly, both DOCK8 patient NK cells 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 [23]. However, Cdc42 is specifically invoked in response to activation, therefore there is a defect in F-actin accumulation at the NK cell synapse of DOCK8 patient NK cells in conjugation with susceptible target cells as measured quantitatively by confocal microscopy (Figure 1B) [23]. 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 process [10] [24]. Granule convergence to the MTOC, which occurs prior to both these steps and was previously reported to be independent of F-actin regulation [25], 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 may be a more general upstream regulator of actin activators such as WASp and WAVE2, or that there may 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 [26].

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 mutation in Coronin 1A as a rare cause of primary of immune deficiency. Coronin 1A promotes F-actin turnover through the regulation of both cofilin (which severs F-actin filaments), and through the negative regulation of the Arp2/3 complex (the actin branch facilitator). Previous immunological defects associated with Coronin 1A mutation in both mouse and human include impaired T cell migration and thymic egress, likely due to misregulation of F-actin [27, 28]. The latter was demonstrated by confocal microscopy in a mouse model of the disease, but not in patient cells owing to an initial scarcity of samples.

A further insight to the effect of Coronin 1A mutation was determined through the application of super-resolution microscopy to the investigation of fine F-actin structure and was observed in patient 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 [29]. 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 [27, 30, 31]. Importantly, discovery of the fine actin structure defect in Coronin 1A deficiency was only enabled by the use of super-resolution imaging, as the defect was not detected by conventional confocal imaging. As such, the discovery represents the first published application of super-resolution imaging in immune cells from PID patients.

The importance of Coronin 1A in F-actin deconstruction for lytic granule access to the plasma membrane complemented earlier work describing the importance of non-muscle myosin IIA in NK cell cytotoxicity. The myosin superfamily, whose members generate ATP-dependent movement along actin filaments, play numerous roles in adhesion, migration and immune function [32]. Mutations in myosin II heavy chain lead to MYH9 related disease (MYH9-RD), which is characterized by hematologic defects, including macrothrombocytopenia, and in some cases cataracts, deafness and glomerulonephritis [33]. Through the use of confocal, 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-RD 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 [34]. 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 [35]. In what was the first application of super-resolution 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, like in Coronin 1A mutated cells lytic granules appear unable to penetrate synaptic actin in the absence of myosin function (Figure 1B), suggesting that myosin may be required for

generating the force required to mobilize relatively large lytic granules across synaptic actin [35-37].

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 be continually approaching 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 response. 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 immunological synapse formation and function. Many of the intricacies of these diseases have been resolved using quantitative imaging.

Integrins bridge the actin cytoskeleton and cell signaling molecules and are critical for immune cell function. In particular, LFA-1 plays an indispensable role in T cell adhesion, migration, extravasation, and scanning of DCs in lymph nodes [38]. LFA-1 provides the crucial adhesive force and actin rearrangement required for immunological synapse 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 [25]. LFA-1 function is tightly regulated by its conformational state and can be rapidly activated by signaling that increases affinity for its primary ligand, ICAM-1. In addition, following ligand binding, LFA-1 undergoes both conformational activation and also mediates signaling itself, leading to dynamic actin rearrangement that is required for cytotoxic function. Finally, LFA-1 plays a critical role in the migration of neutrophils and platelet adhesion. LAD-1 patients 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 LAD-I cells are limited to the cell biology of NK cells, in which LFA-1 functions as an early signal for lytic granule convergence to the MTOC prior to MTOC reorientation and directed secretion of granules [25]. Using fixed cell confocal microscopy, it was shown that LAD-I patient NK cells fail to converge lytic granules to the MTOC following conjugation with susceptible target cells [39]. As 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 patient NK cells in cytotoxicity assays. Adhesion to target cells, while decreased at early time points, was not significantly impaired by 60 minutes in LAD-I patient cells. 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 LAD-1 NK cells 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. While Ca^{2+} is a well-documented second messenger, roles for Mg^{2+} and Zn^{2+} as important signaling molecules are now also known [40]. The importance of cation signaling is underscored by the discovery of primary immune deficiency that occurs as a result of mutations in Mg^{2+} or Ca^{2+} channels. In the case of Mg^{2+} signaling, mutations in the MAGT1 gene result in X-MEN disease (X-linked immunodeficiency with Mg^{2+} defect, EBV infection and neoplasia) [41]. 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 due to decreased cell surface expression of the important activating receptor NKG2D, which was also decreased on T cells and even EBV transformed B cell lines from patients. This was demonstrated in part using quantitative confocal microscopy. The presence of exogenous Mg^{2+} supplemented either in cell media, or impressively as an oral therapy in patients, restored NKG2D levels on patient cells as well as effector cell function [41]. The signaling leading from the T cell receptor (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. Stores operated calcium entry is required for T cell and NK cell activation, sustained signaling, and exocytosis of lytic granules [41]. Mutations in ORAI1 or STIM1, both of which are required for stores operated calcium entry in NK and T cells, results in impaired NK cell degranulation and cytotoxicity [42]. In NK cells at least this effect seems to be primarily on the relatively late degranulation step, as confocal microscopy of NK cells from ORAI1-deficient patients showed normal granule convergence and polarization [42].

The NF κ B 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/BAFFR/lymphotoxin β receptor leads to non-canonical pathway activation and the NIK-dependent, NEMO-independent processing of p100 to p52 and its subsequent nuclear translocation. While not commonly associated with NK cell activation signaling, the canonical NF κ B pathway is rapidly induced following ligation of the NK cell activating receptor NKp30 [43]. This likely and at least partially accounts for the defect in NK cell cytotoxicity in patients with IKK/NEMO mutations [44]. 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 [43]. Recently, loss-of-function mutations in NF κ B inducing kinase (NIK) leading to novel PID were described [45]. Loss of NIK led to defects in both canonical and non-canonical NF κ B

signaling and included defects in B cell class switching and T cell responses. There was also impaired NK cell function in NIK deficiency, which was analyzed by 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 to healthy controls 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.

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

Intracellular trafficking defects

Modern microscopy techniques applied to PID have had perhaps had the greatest impact in the understanding of defects in cytotoxicity mediated both by T and NK cells. This immune effector function is dependent upon dual-function secretory lysosomes that function as organelles specialized both for lysosomal function and the secretion of perforin and granzymes (Figure 2A). This specialized secretory lysosome function is also utilized in pigment-producing melanocytes via 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 may 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 [22, 46-48]. Frequently, mutations in these pathways cause hemophagocytic lymphohistiocytosis (HLH) due to abrogation of cytotoxicity without impairment of proinflammatory function.

The use of quantitative imaging has proven essential in elucidating both the biology and disease-causing aspects of genetic mutations leading to Griscelli Syndrome 2 (Rab27a), Hermansky-Pudlik Syndrome (AP3), familial hemophagocytic lymphohistiocytosis (FHL) (perforin, Munc13-2, Munc 18-4, syntaxin 11), immune deficiency as a result of p14 mutation (p14) and Chediak-Higashi syndrome (LYST) (Figure 2B).

Albinism and giant lytic granules in immune effector cells accompany Chediak-Higashi, Griscelli and HPS2 syndromes, as all diseases affect the common granule trafficking pathway shared between immune effectors and melanocytes. Prior to the identification of mutations in LYST being causative for CHS and the *beige* mutation in mice, the early observation was made by confocal microscopy that giant lysosomes accumulated in CTL and did not undergo normal exocytosis [49]. This led to the conclusion that the immune defect in CHS at least partially arose from loss of secretion in CTL and raised the hypothesis that the protein involved was involved in vesicle membrane fusion or exocytosis [49]. Interestingly, lysosomes are formed normally in CHS then increase in size due to fusion, or abnormal fission, of lytic granules as shown by electron microscopy (in naïve in vitro

activated CTL clones from CHS patients) [49, 50]. Ultimately, this process results in 1-3 giant lysosomes per cell [49, 50]. 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 [51-53] which is now more clearly ascribed to the generation of individual secretory lysosomes and, particularly, the terminal maturation of perforin-containing granules into exocytosis competent granules [54]. These elegant new studies performed in part using TIRF, confocal and correlative light electron microscopy show that enlarged granules result from the fusion of normal sized endo-lysosomal 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 [54]. Finally, the overexpression of effectors involved in degranulation was able to restore the ability of CHS CTL 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 due to 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 HPS2 CTL based on confocal microscopy studies of granule components [55]. Specifically, CTL clones derived from HPS2 patients were stained with antibodies to lytic granule components and showed strong co-localization of both LAMP2 and perforin with Cathepsin D, indicating appropriate localization of lytic granule constituents. Interestingly, the well-defined defect in CTL cytotoxicity in HPS2 was described, also by confocal microscopy, to be due to impaired transit of lytic granules along microtubules, resulting in defective polarized secretion. When examined by TEM, HPS2 granules are also enlarged and contained many more multivesicular structures in comparison to healthy normal donor granules, which upon maturation have an electron dense core [50, 55, 56]. NK cell cytotoxicity is also impaired in HPS2 patients and although HPS2 NK cells have not been specifically described by microscopy, it is thought that AP3 is playing a similar role in NK cells [57]. While the exact mechanism of this is unknown, it may be that a second protein required for lytic granule attachment to, or movement on, microtubules is inappropriately localized in the absence of AP3.

While Griscelli syndrome 2 (GS2) is also associated with albinism, the mutation of Rab27a results in normal granule polarization but a defect in lytic granule docking and subsequent degranulation based on confocal microscopy studies done in mouse CTL and Rab27a deficient NK cell lines [58-61]. In NK cells, ligation of activating receptor recruits both Rab27a and Munc13-4 to lytic granules, which was shown by measuring their co-localization with perforin in confocal microscopy images [62]. NK cells from a GS2 patient were impaired in their ability to recruit Munc13-4 to granules, suggesting that Rab27a is required for assembling the secretory lysosome docking complex [62].

UNC13D, which encodes Munc13-4, is mutated in FHL type 3. Both NK and CTL have defective cytotoxic function in these patients, despite demonstrating normal lytic granule polarization towards target cells as visualized by confocal microscopy and TEM [63, 64]. 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 via confocal microscopy, which suggests that the role of Munc13-4 may also be to recruit and prime Rab27a [65, 66]. 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 FHL type 4, which is caused by mutations in the *STX11* gene [63]. Similar to the previously described docking and priming defects, Syntaxin-11-deficient NK cells have normal granule polarization towards the target cell, as demonstrated by confocal microscopy of FHL4 patient cells [67]. Munc18-2, which is encoded by the *STXBP2* gene, is mutated in FHL type 5. Munc18-2 colocalizes with Syntaxin 11 as measured in quantitative imaging experiments. Confocal microscopy using FHL5 patient NK cells again show normal lytic granule polarization, but defective degranulation [68]. 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 p14 human disease was defined as causing PID with partial albinism, short stature, congenital neutropenia and B- and T-cell lymphopenia and a form of HLH [69]. Further studies defined via 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 the neutrophils of CHS patients 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 and suggests some more inherent abnormality in the granules themselves in 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 has been used to richly define genetic disorders affecting the cellular host defenses against pathogens provided by neutrophils and macrophages. This particularly includes those affecting a visually striking and critical role of neutrophils, that of the 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 [70-72]. The discovery of NETs would not have been possible were it not for extraordinary attention to detail using high resolution imaging, as these delicate and enigmatic structures were initially very difficult to characterize 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 acquired neutrophil dysfunction disorders and hereditary disorders of unknown etiology.

The initial description of neutrophil NETs was a microscopy-driven discovery based on the surprising observation that activated neutrophils produced fragile, yet visible, extracellular projections when visualized by both electron and immunofluorescence microscopy [70]. 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 following DNase treatment and in situ immunohistochemistry showing the presence of NETs in tissues. The generation of NETs is dependent upon the generation of reactive oxygen species, following which neutrophil elastase and myeloperoxidase trigger the decondensation of chromatin and the subsequent release of NETs into extracellular space (Figure 3A). Due to this dependence on ROS, NET formation in CGD patients is also impaired [73] (Figure 3B). This was defined using confocal microscopy of neutrophils isolated from CGD patients. Interestingly, CGD patient neutrophils retain the ability to form NETs when stimulated with glucose oxidase [73], however after infection with *S. aureus* NET formation was impaired. Both these clinically relevant observations were made using immunostaining of neutrophils isolated from CGD patients.

In CGD, mutations in any one of five NADPH oxidase subunits (membrane-bound gp91^{phox} and p22^{phox} and cytosolic p47^{phox}, p67^{phox} and p40^{phox}) can impair reactive oxygen species (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 [74, 75]. Activation of the pathway results in the binding of the cytoplasmic components to each other, followed by their translocation, with the GTPase Rac, to the plasma membrane and association with the membrane bound components and, likely, the actin cytoskeleton [76-80]. The subcellular localization of these components was intricately defined by the combination of microscopy with biochemical phagocytosis assays to detect bacterial opsonization [81-83]. Loss of gp91^{phox} most likely affects complex formation through the inability of the cytosolic complex to dock at the plasma membrane, and studies using p47^{phox} deficient individuals show that p67 recruitment requires p47^{phox} [81]. p47^{phox} and p67^{phox} are recruited normally to phagosomes in both normal and CGD PMNs, however 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 using fixed cell fluorescence microscopy of adherent neutrophils opsonizing bacteria. Following 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 co-localization 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 normal, but not CGD PMN. Relatively straightforward scoring of positive vs. 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 the use of microscopy.

Gene therapy to restore gp91^{phox} expression may be an option for treatment in certain CGD patients, as expression of a monocistronic long terminal repeat-driven gamma-retroviral SF71-gp91^{phox} vector in a boy with X-linked CGD resulted in the visible increase in NET formation and clearance of aspergillus hyphae following in vitro infection of the patient's neutrophils with aspergillus isolated from his own disease [84]. 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 CGD patient cells is the use of liposomes to deliver glucose oxidase [85]. Confocal and TEM microscopy show the uptake of bacteria and liposome-derived particles and treatment with glucose-oxidase containing liposomes increased the killing of *S. aureus* by CGD granulocytes.

Finally, although utilizing a mouse model of CGD, the importance of CD4+ T cells in the control of *aspergillus* infection was illustrated using confocal microscopy and underscores the role of the NADPH pathway in DC cross-priming of T cells [86]. This clearly demarcated the intracellular pathway of processing of *aspergillus* conidia by confocal staining for lysosomal pathway components and fluorescently labeled fungal antigen. This showed impaired lysosomal localization in CGD patient DCs and resulting impaired T cell response. Thus through advanced microscopy-based subcellular localization study in PID mechanistic insight was gained into a perplexing abnormality present in T cells in CGD patients.

Conclusion

An increase in the technology available for rapid and accurate diagnosis of primary immune deficiency has resulted in a correlative increase in the opportunity for important cell biological discovery that can be made in parallel. With the advent of relatively accessible whole exome sequencing, chromosomal array and the bioinformatics analyses to support these technologies, the genetic cause for disease in rare primary immune deficiencies 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 immunological and cell biological phenotype in afflicted individuals 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 the progression towards the common goal of therapeutic advances in the treatment of these unique patients.

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References

1. Moulding DA, et al. Actin cytoskeletal defects in immunodeficiency. *Immunol Rev.* 2013; 256(1): 282–99. [PubMed: 24117828]
2. Mace EM, et al. Cell biological steps and checkpoints in accessing NK cell cytotoxicity. *Immunol Cell Biol.* 2014; 92(3):245–55. [PubMed: 24445602]
3. Massaad MJ, Ramesh N, Geha RS. Wiskott-Aldrich syndrome: a comprehensive review. *Ann N Y Acad Sci.* 2013; 1285:26–43. [PubMed: 23527602]
4. Derry JM, Ochs HD, Francke U. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell.* 1994; 78(4):635–44. [PubMed: 8069912]
5. Remold-O'Donnell E, Rosen FS, Kenney DM. Defects in Wiskott-Aldrich syndrome blood cells. *Blood.* 1996; 87(7):2621–31. [PubMed: 8639877]
6. Gallego MD, et al. Defective actin reorganization and polymerization of Wiskott-Aldrich T cells in response to CD3-mediated stimulation. *Blood.* 1997; 90(8):3089–97. [PubMed: 9376590]
7. Molina IJ, et al. T cell lines characterize events in the pathogenesis of the Wiskott-Aldrich syndrome. *J Exp Med.* 1992; 176(3):867–74. [PubMed: 1512549]
8. Westerberg L, et al. Cdc42, Rac1, and the Wiskott-Aldrich syndrome protein are involved in the cytoskeletal regulation of B lymphocytes. *Blood.* 2001; 98(4):1086–94. [PubMed: 11493455]
9. Symons M, et al. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell.* 1996; 84(5):723–34. [PubMed: 8625410]
10. Orange JS, et al. Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. *Proc Natl Acad Sci U S A.* 2002; 99(17):11351–6. [PubMed: 12177428]
11. De Meester J, et al. The Wiskott-Aldrich syndrome protein regulates CTL cytotoxicity and is required for efficient killing of B cell lymphoma targets. *J Leukoc Biol.* 2010; 88(5):1031–40. [PubMed: 20689099]
12. Sims TN, et al. Opposing effects of PKC θ and WASp on symmetry breaking and relocation of the immunological synapse. *Cell.* 2007; 129(4):773–85. [PubMed: 17512410]
13. Orange JS, et al. IL-2 induces a WAVE2-dependent pathway for actin reorganization that enables WASp-independent human NK cell function. *J Clin Invest.* 2011; 121(4):1535–48. [PubMed: 21383498]
14. Gismondi A, et al. Impaired natural and CD16-mediated NK cell cytotoxicity in patients with WAS and XLT: ability of IL-2 to correct NK cell functional defect. *Blood.* 2004; 104(2):436–43. [PubMed: 15001467]
15. Nolz JC, et al. The WAVE2 complex regulates actin cytoskeletal reorganization and CRAC-mediated calcium entry during T cell activation. *Curr Biol.* 2006; 16(1):24–34. [PubMed: 16401421]
16. Boztug K, et al. Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N Engl J Med.* 2010; 363(20):1918–27. [PubMed: 21067383]
17. Aiuti A, et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science.* 2013; 341(6148):1233151. [PubMed: 23845947]
18. Machlus KR, Italiano JE Jr. The incredible journey: From megakaryocyte development to platelet formation. *J Cell Biol.* 2013; 201(6):785–96. [PubMed: 23751492]
19. Haddad E, et al. The thrombocytopenia of Wiskott Aldrich syndrome is not related to a defect in proplatelet formation. *Blood.* 1999; 94(2):509–18. [PubMed: 10397718]
20. Taylor MD, et al. Nuclear role of WASp in the pathogenesis of dysregulated TH1 immunity in human Wiskott-Aldrich syndrome. *Sci Transl Med.* 2010; 2(37):37ra44.
21. Engelhardt KR, et al. Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. *J Allergy Clin Immunol.* 2009; 124(6):1289–302 e4. [PubMed: 20004785]
22. Orange JS. Natural killer cell deficiency. *J Allergy Clin Immunol.* 2013; 132(3):515–25. quiz 526. [PubMed: 23993353]

23. Mizesko MC, et al. Defective actin accumulation impairs human natural killer cell function in patients with dedicator of cytokinesis 8 deficiency. *J Allergy Clin Immunol.* 2013; 131(3):840–8. [PubMed: 23380217]
24. Orange JS, et al. The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proc Natl Acad Sci U S A.* 2003; 100(24):14151–6. [PubMed: 14612578]
25. Mentlik AN, et al. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Mol Biol Cell.* 2010; 21(13):2241–56. [PubMed: 20444980]
26. Ham H, et al. Dedicator of cytokinesis 8 interacts with talin and Wiskott-Aldrich syndrome protein to regulate NK cell cytotoxicity. *J Immunol.* 2013; 190(7):3661–9. [PubMed: 23455509]
27. Shiow LR, et al. Severe combined immunodeficiency (SCID) and attention deficit hyperactivity disorder (ADHD) associated with a Coronin-1A mutation and a chromosome 16p11.2 deletion. *Clin Immunol.* 2009; 131(1):24–30. [PubMed: 19097825]
28. Shiow LR, et al. The actin regulator coronin 1A is mutant in a thymic egress-deficient mouse strain and in a patient with severe combined immunodeficiency. *Nat Immunol.* 2008; 9(11):1307–15. [PubMed: 18836449]
29. Mace EM, Orange JS. Lytic immune synapse function requires filamentous actin deconstruction by Coronin 1A. *Proc Natl Acad Sci U S A.* 2014; 111(18):6708–13. [PubMed: 24760828]
30. Moshous D, et al. Whole-exome sequencing identifies Coronin-1A deficiency in 3 siblings with immunodeficiency and EBV-associated B-cell lymphoproliferation. *J Allergy Clin Immunol.* 2013; 131(6):1594–603. [PubMed: 23522482]
31. Stray-Pedersen A, et al. Compound heterozygous CORO1A mutations in siblings with a mucocutaneous-immunodeficiency syndrome of epidermodysplasia verruciformis-HPV, molluscum contagiosum and granulomatous tuberculoid leprosy. *J Clin Immunol.* 2014; 34(7):871–90. [PubMed: 25073507]
32. Hartman MA, Spudich JA. The myosin superfamily at a glance. *J Cell Sci.* 2012; 125(Pt 7):1627–32. [PubMed: 22566666]
33. Seri M, et al. MYH9-related disease: May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, and Epstein syndrome are not distinct entities but represent a variable expression of a single illness. *Medicine (Baltimore).* 2003; 82(3):203–15. [PubMed: 12792306]
34. Andzelm MM, et al. Myosin IIA is required for cytolytic granule exocytosis in human NK cells. *J Exp Med.* 2007; 204(10):2285–91. [PubMed: 17875677]
35. Sanborn KB, et al. Phosphorylation of the myosin IIA tailpiece regulates single myosin IIA molecule association with lytic granules to promote NK-cell cytotoxicity. *Blood.* 2011; 118(22):5862–71. [PubMed: 22123909]
36. Mace EM, et al. NK cell lytic granules are highly motile at the immunological synapse and require F-actin for post-degranulation persistence. *J Immunol.* 2012; 189(10):4870–80. [PubMed: 23066148]
37. Sanborn KB, et al. Myosin IIA associates with NK cell lytic granules to enable their interaction with F-actin and function at the immunological synapse. *J Immunol.* 2009; 182(11):6969–84. [PubMed: 19454694]
38. Hogg N, Patzak I, Willenbrock F. The insider's guide to leukocyte integrin signalling and function. *Nat Rev Immunol.* 2011; 11(6):416–26. [PubMed: 21597477]
39. James AM, et al. Rapid activation receptor- or IL-2-induced lytic granule convergence in human natural killer cells requires Src, but not downstream signaling. *Blood.* 2013; 121(14):2627–37. [PubMed: 23380740]
40. Chaigne-Delalande B, Lenardo MJ. Divalent cation signaling in immune cells. *Trends Immunol.* 2014; 35(7):332–44. [PubMed: 24932518]
41. Chaigne-Delalande B, et al. Mg²⁺ regulates cytotoxic functions of NK and CD8 T cells in chronic EBV infection through NKG2D. *Science.* 2013; 341(6142):186–91. [PubMed: 23846901]
42. Maul-Pavicic A, et al. ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis. *Proc Natl Acad Sci U S A.* 2011; 108(8):3324–9. [PubMed: 21300876]

43. Pandey R, et al. NKp30 ligation induces rapid activation of the canonical NF-kappaB pathway in NK cells. *J Immunol.* 2007; 179(11):7385–96. [PubMed: 18025182]
44. Orange JS, et al. Deficient natural killer cell cytotoxicity in patients with IKK-gamma/NEMO mutations. *J Clin Invest.* 2002; 109(11):1501–9. [PubMed: 12045264]
45. Willmann KL, et al. Biallelic loss-of-function mutation in NIK causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity. *Nat Commun.* 2014; 5:5360. [PubMed: 25406581]
46. de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol.* 2010; 10(8):568–79. [PubMed: 20634814]
47. Ham H, Billadeau DD. Human immunodeficiency syndromes affecting human natural killer cell cytolytic activity. *Front Immunol.* 2014; 5:2. [PubMed: 24478771]
48. Krzewski K, Coligan JE. Human NK cell lytic granules and regulation of their exocytosis. *Front Immunol.* 2012; 3:335. [PubMed: 23162553]
49. Baetz K, Isaaz S, Griffiths GM. Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. *J Immunol.* 1995; 154(11):6122–31. [PubMed: 7751653]
50. Stinchcombe JC, Page LJ, Griffiths GM. Secretory lysosome biogenesis in cytotoxic T lymphocytes from normal and Chediak Higashi syndrome patients. *Traffic.* 2000; 1(5):435–44. [PubMed: 11208129]
51. Barbosa MD, et al. Identification of the homologous beige and ChediakHigashi syndrome genes. *Nature.* 1996; 382(6588):262–5. [PubMed: 8717042]
52. Fukai K, et al. Homozygosity mapping of the gene for Chediak-Higashi syndrome to chromosome 1q42-q44 in a segment of conserved synteny that includes the mouse beige locus (bg). *Am J Hum Genet.* 1996; 59(3):620–4. [PubMed: 8751863]
53. Nagle DL, et al. Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. *Nat Genet.* 1996; 14(3):307–11. [PubMed: 8896560]
54. Sepulveda FE, et al. LYST controls the biogenesis of the endosomal compartment required for secretory lysosome function. *Traffic.* 2014
55. Clark RH, et al. Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. *Nat Immunol.* 2003; 4(11):1111–20. [PubMed: 14566336]
56. Ward DM, et al. Analysis of the lysosomal storage disease ChediakHigashi syndrome. *Traffic.* 2000; 1(11):816–22. [PubMed: 11208072]
57. Orange JS. Formation and function of the lytic NK-cell immunological synapse. *Nat Rev Immunol.* 2008; 8(9):713–25. [PubMed: 19172692]
58. Haddad EK, et al. Defective granule exocytosis in Rab27a-deficient lymphocytes from Ashen mice. *J Cell Biol.* 2001; 152(4):835–42. [PubMed: 11266473]
59. Liu D, Meckel T, Long EO. Distinct role of rab27a in granule movement at the plasma membrane and in the cytosol of NK cells. *PLoS One.* 2010; 5(9):e12870. [PubMed: 20877725]
60. Menasche G, et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genet.* 2000; 25(2):173–6. [PubMed: 10835631]
61. Stinchcombe JC, et al. Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *J Cell Biol.* 2001; 152(4):825–34. [PubMed: 11266472]
62. Wood SM, et al. Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity. *Blood.* 2009; 114(19):4117–27. [PubMed: 19704116]
63. Feldmann J, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell.* 2003; 115(4):461–73. [PubMed: 14622600]
64. Marcenaro S, et al. Analysis of natural killer-cell function in familial hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. *Blood.* 2006; 108(7):2316–23. [PubMed: 16778144]

65. Neeft M, et al. Munc13-4 is an effector of rab27a and controls secretion of lysosomes in hematopoietic cells. *Mol Biol Cell*. 2005; 16(2):731–41. [PubMed: 15548590]
66. Shirakawa R, et al. Munc13-4 is a GTP-Rab27-binding protein regulating dense core granule secretion in platelets. *J Biol Chem*. 2004; 279(11):10730–7. [PubMed: 14699162]
67. Bryceson YT, et al. Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. *Blood*. 2007; 110(6):1906–15. [PubMed: 17525286]
68. Cote M, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J Clin Invest*. 2009; 119(12):3765–73. [PubMed: 19884660]
69. Bohn G, et al. A novel human primary immunodeficiency syndrome caused by deficiency of the endosomal adaptor protein p14. *Nat Med*. 2007; 13(1):38–45. [PubMed: 17195838]
70. Brinkmann V, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004; 303(5663):1532–5. [PubMed: 15001782]
71. Saitoh T, et al. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe*. 2012; 12(1):109–16. [PubMed: 22817992]
72. Urban CF, et al. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol*. 2006; 8(4):668–76. [PubMed: 16548892]
73. Fuchs TA, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007; 176(2):231–41. [PubMed: 17210947]
74. Reeves EP, et al. Killing activity of neutrophils is mediated through activation of proteases by K⁺ flux. *Nature*. 2002; 416(6878):291–7. [PubMed: 11907569]
75. Tkalcevic J, et al. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. *Immunity*. 2000; 12(2):201–10. [PubMed: 10714686]
76. Dusi S, et al. Mechanisms of stimulation of the respiratory burst by TNF in nonadherent neutrophils: its independence of lipidic transmembrane signaling and dependence on protein tyrosine phosphorylation and cytoskeleton. *J Immunol*. 1996; 157(10):4615–23. [PubMed: 8906841]
77. el Benna J, Ruedi JM, Babior BM. Cytosolic guanine nucleotide-binding protein Rac2 operates in vivo as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47phox and p67phox to the submembranous actin cytoskeleton during oxidase activation. *J Biol Chem*. 1994; 269(9):6729–34. [PubMed: 8120032]
78. Hall A. Ras-related GTPases and the cytoskeleton. *Mol Biol Cell*. 1992; 3(5):475–9. [PubMed: 1611153]
79. Leusen JH, et al. Disturbed interaction of p21-rac with mutated p67-phox causes chronic granulomatous disease. *J Exp Med*. 1996; 184(4):1243–9. [PubMed: 8879195]
80. Woodman RC, et al. Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *J Clin Invest*. 1991; 87(4):1345–51. [PubMed: 1849148]
81. Allen LA, et al. Transient association of the nicotinamide adenine dinucleotide phosphate oxidase subunits p47phox and p67phox with phagosomes in neutrophils from patients with X-linked chronic granulomatous disease. *Blood*. 1999; 93(10):3521–30. [PubMed: 10233905]
82. Clark RA, et al. Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. *N Engl J Med*. 1989; 321(10):647–52. [PubMed: 2770793]
83. Curnutte JT. Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. *Clin Immunol Immunopathol*. 1993; 67(3 Pt 2):S2–15. [PubMed: 8500277]
84. Bianchi M, et al. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood*. 2009; 114(13):2619–22. [PubMed: 19541821]
85. Gerber CE, et al. Reconstitution of bactericidal activity in chronic granulomatous disease cells by glucose-oxidase-containing liposomes. *Blood*. 2001; 98(10):3097–105. [PubMed: 11698296]
86. De Luca A, et al. CD4(+) T cell vaccination overcomes defective cross-presentation of fungal antigens in a mouse model of chronic granulomatous disease. *J Clin Invest*. 2012; 122(5):1816–31. [PubMed: 22523066]

87. Bianchi M, et al. Restoration of anti-Aspergillus defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calprotectin-dependent. *J Allergy Clin Immunol.* 2011; 127(5):1243–52 e7. [PubMed: 21376380]
88. Martin-Cofreces NB, Baixauli F, Sanchez-Madrid F. Immune synapse: conductor of orchestrated organelle movement. *Trends Cell Biol.* 2014; 24(1):61–72. [PubMed: 24119664]

Figure 1. Effect of primary immune deficiency on the actin cytoskeleton

A) Pathway leading to F-actin polymerization and structural rearrangement at the immune synapse. Upon 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. B) Effect of primary immune deficiency affecting the actin cytoskeleton as measured by high-resolution microscopy. i) transmission electron micrograph of F-actin at the NK cell immune synapse from a healthy donor (left) or WAS patient (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. MYH9-RD patients suffer NK cell deficiency due to 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 from the peripheral blood of a WAS patient treated by 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 to 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 Coro1AKD human NK cell line (bottom) compared to the control line (top). Cells were fixed and stained for F-actin (green) and lytic granules (red) then visualized in the XZ plane by STED microscopy.

Syndrome	Gene/protein	Effect on granules visualized by microscopy	Albinism	Reference (microscopy)
Chediak-Higashi Syndrome	<i>LYST/</i> <i>LYST</i>	Giant lytic granules	Yes (partial)	[49], [50], [54]
Hermansky-Pudlik Syndrome type 2	<i>AP3B1/</i> <i>AP3</i>	Enlarged granules, impaired polarization	Yes	[55]
FHL type 2	<i>PRF1/</i> perforin	Normal granule polarization	No	[64]
FHL type 3	<i>UNC13D/</i> Munc13-4	Normal granule polarization	No	[63], [64]
FHL type 4	<i>STX11/</i> Syntaxin 11	Normal granule polarization	No	[67]
FHL type 5	<i>STXBP2/</i> Munc18-2	Normal granule polarization	No	[68]
Griscelli Syndrome type 2	<i>RAB27A/</i> Rab27a	Giant lytic granules; impaired recruitment of Munc13-4 to granules	Yes	[62]

Figure 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 prior to 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 GFP variant to the neutral extracellular environment. Image was acquired by TIRF microscopy at the plasma membrane of an NK cell activated on glass. B) Table of primary immune deficiencies 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.

Figure 3. Cellular host defense through the formation of NETs

A) NET formation requires both phagosome formation and reactive oxygen species (ROS).

B) NET formation is impaired in neutrophils from CGD patients. Control (left) or patient (right) neutrophils were stimulated with PMA and ionomycin and imaged 5h later by scanning electron microscopy. Scale bar=10 mm. Image courtesy of Dr. Janine Reichenbach, University Children's Hospital Zurich.

Table 1
High-resolution imaging techniques utilized in cell biological studies of primary immune deficiency including references to use in Primary Immune Deficiency

Name	Description	PID reference
<i>Confocal microscopy</i>	The use of spatially aligned pinhole to improve resolution of fluorescence microscopy through the exclusion of out-of-focus light.	[10-13, 16, 17, 23, 24, 35, 37, 39, 41-43, 45, 49, 54, 55, 62-69, 73, 84, 85]
<i>Super-resolution microscopy</i>	The use of optics or mathematical algorithms to achieve resolution below the diffraction limit of light.	[29, 35]
<i>Fluorescence widefield</i>	The use of whole specimen illumination to visualize structures labeled with fluorophores.	[19, 20, 81]
<i>Transmission electron microscopy (TEM)</i>	The use of a focused beam of electrons passed through an ultra-thin specimen to illuminate structural elements.	[5, 6, 8, 13, 35, 50, 55, 63, 73, 85, 87]
<i>Total internal reflection microscopy (TIRF)</i>	The use of an evanescent wave to selectively illuminate the membrane proximal 150 nm of cell.	[54]
<i>Imaging flow cytometry (IFC)</i>	The combination of conventional flow cytometry with fluorescence microscopy to assess both population based phenotype and protein localization.	
<i>Correlative light electron microscopy (CLEM)</i>	The use of light microscopy of fixed cells followed by the imaging of structures by electron microscopy.	[54]

Table 2
Key cellular structures affected by Primary Immune Deficiency

Structure	Description	Size	Reference (review)
<i>MTOC</i>	Centrosome; facilitates delivery of lytic granules to the immune synapse		[88]
<i>F-actin</i>	Smallest cytoskeletal filament; promotes adhesion and migration	5-10 nm	[1]
<i>Microtubules</i>	Largest cytoskeletal filament; framework for transport of lytic granules and other cargo	15-20 nm	[88]
<i>Lytic granules</i>	Specialized acidified lysosomes containing perforin and granzymes	100-750 nm	[46]