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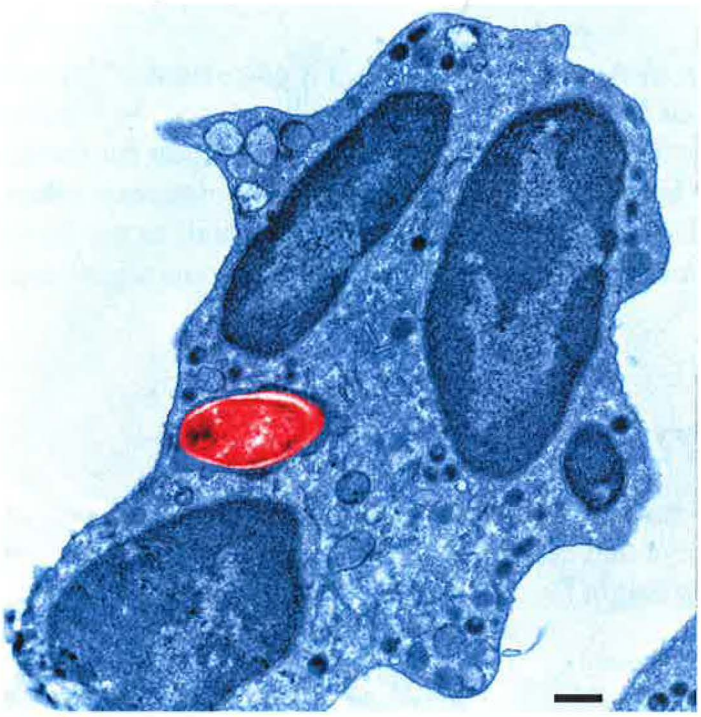


Save the Canary: When to Suspect Phagocyte Disorders

David Greenberg, MD

Internal Medicine Grand Rounds
University of Texas Southwestern Medical Center

This is to acknowledge that David Greenberg, MD has disclosed a financial interest or other relationships with commercial concerns related directly or indirectly to this program. Dr. Greenberg will not be discussing off-label uses in his presentation.

Biographical Information

Dr. Greenberg is an Assistant Professor in the Division of Infectious Diseases and the Department of Microbiology. He is the director of the Medical Microbiology course during the MS2 year and the Associate Director for the adult ID Fellowship program here at UTSW. Dr. Greenberg's research interests relate to host-pathogen interactions in the immunocompromised host as well as the development of novel therapeutic approaches for medically important Gram-negative pathogens.

Purpose & Overview

The goal of this session is to better understand genetic causes of phagocyte disorders and ways that certain pathogens can give clues as to what the defective immune pathway might be.

Educational Objectives

1. Recognize that genetic disorders of phagocytes can present and ultimately be diagnosed in adulthood.

2. Recognize the role that neutrophils and macrophages play in host defense.

3. Recognize that the pathogen a particular patient may present with can give clues as to the underlying genetic defect.

Introduction

Phagocytes serve as the bedrock of the innate immune system. The two primary phagocytic cells, neutrophils and macrophages, have both overlapping and unique roles in preventing microbial invaders from gaining access to areas of the human body where they may cause damage or "infection". It has long been recognized that phagocytes were one of the "first lines of defense" against pathogens. Elias Metchnikoff eloquently demonstrated the influx of these cells to areas of damage (Figure 1) and thought these cells would play a role in the digestion of microbes[1]. This protocol reviews some of the prototypic disorders of phagocytic function. These experiments of nature shed light on the pathways that are critical for normal functioning of the innate immune system.

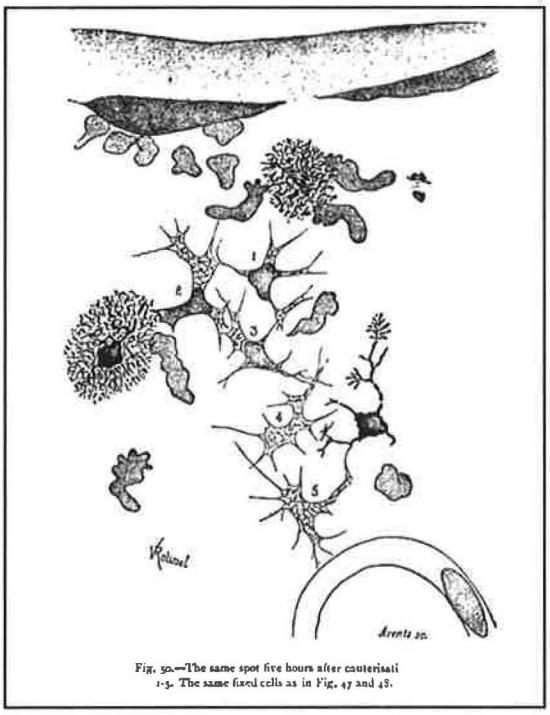


Fig. 50.—The same spot five hours after cauterisati
1-5. The same fixed cells as in Fig. 47 and 48.

Figure 1. Drawing of phagocytes arriving at the site of injury. Metchnikoff 1891.

A number of warning signs in the medical history can point to a primary immunodeficiency (Table 1)[2]. In many cases, just as the "canary in the coal mine" is the sentinel that warns miners of toxic gases, the pathogen with which the patient presents can be the warning sign that guides the physician to the immune pathway that is perturbed.

Medical history
Recurrent (proven) bacterial infections
Two ore more severe infections (pneumonia, sepsis, meningitis, osteomyelitis)
Atypical presentation of infection
Unusually severe course of infection or impaired response to treatment
Infections caused by an unexpected or opportunistic pathogen
Recurrent infections with the same type of pathogen
Abscesses of internal organs or recurrent subcutaneous abscesses
Failure to thrive with prolonged or recurrent diarrhea
Generalized long-lasting warts or mollusca contagiosa
Extensive prolonged candidiasis (oral/skin)
Delayed (>4 weeks) separation of the umbilical cord
Delayed shedding of primary teeth
Family history of immunodeficiency, unexplained infant deaths, or consanguinity of the parents
Difficult-to-treat obstructive lung disease, unexplained bronchiectasis
Atypical autoimmune disease and/or lymphoproliferation

Table 1. Historical features suggestive of a primary immunodeficiency (PID)

Normal Neutrophil Function

While most primary disorders of neutrophils are individually quite rare, most physicians recognize the importance of the polymorphonuclear leukocyte (PMN) or neutrophil in innate immune defense. Years of experience with the administration of chemotherapeutic agents, which can dramatically wipe out our neutrophils, clearly demonstrate the infectious consequences that can ensue[3]. Neutrophils are the most abundant leukocytes in the blood, comprising 60-70% of all the leukocytes present. In addition, they exist in very high numbers (~5 x 10^9 cells/liter). Interestingly, the cells are short lived, circulating for only 6-8 hours at a time[4]. The neutrophil is well equipped to deal with pathogens, and contains numerous substances that are toxic to microbes (as well as to the host on occasion)(Figure 2)[5]. These include specialized storage compartments or granules that contain a plethora of proteins that have direct effects on microbes (Table 2). As a neutrophil is activated (as during phagocytosis) these granules become mobilized[4, 6] and then fuse with either the phagosome or the plasma membrane to release their contents. Many of the proteins contained within these granules are directly toxic to the microbes and represent what are generally referred to as oxygen-independent antimicrobial mechanisms. Perhaps equally important are the oxygen-dependent antimicrobial mechanisms of the neutrophil, carried out by the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase complex[7]. The complex is composed of at least six components. The flavocytochrome b_{558} contains $gp91^{phox}$ and $gp22^{phox}$ and resides mainly on the Secondary (specific) granules as well as on the plasma membrane[8]. Upon stimulation, the cytosolic components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac2) join with the membrane components and together form the NADPH oxidase complex[9] that produces the "respiratory burst" of the neutrophil resulting in the subsequent production of superoxide and other toxic oxygen metabolites (i.e. H_2O_2, HOCL).

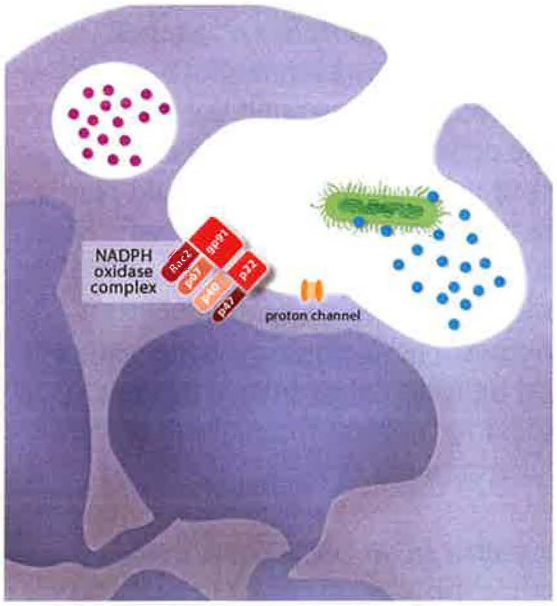


Figure 2. Graphic representation of a neutrophil phagocytosing a pathogen. The NADPH complex is assembling and granules are fusing with the developing phagosome.

Table 2. Components and Antimicrobial Effect of Neutrophil Granule Components

Antimicrobial peptide	Antimicrobial mechanism
Cationic antimicrobial peptides	
α-defensins	Permeabilize membrane bilayers Inhibit DNA, RNA biosynthesis Inhibit bacterial cell wall synthesis
LL-37	Transmembrane pore-forming
BPI	Increases bacterial permeability by binding to LPS
Histones	Unknown
Proteolytic enzymes	
Lysozyme	Degrades bacterial cell wall
Proteinase 3	Binds to bacterial membrane
Neutrophil elastase (NE), cathepsin G (CG)	Cleaves outer membrane proteins and bacterial virulence factors
Azurocidin	
Metal chelator proteins	
Lactoferrin	Binds iron, an essential bacterial nutrient Increases membrane permeability
Calprotectin	Sequesters manganese and zinc

Disorders of Neutrophil Function

There are a variety of congenital disorders of neutrophil function[10]. Although many of them first present with clinical manifestations during early childhood, the underlying genetic defect is often not diagnosed until adulthood. However, in some cases, the first serious clinical manifestations do not occur until adulthood[11]. These disorders span the entire life cycle of the neutrophil, including problems with bone marrow egress[12-14], neutrophil adhesion[15], defects in microbial killing and defects in chemotactic ability [16] (Table 3). While more frequent disorders such as myeloperoxidase deficiency (estimated prevalence of 1:2000-1:4000 in the U.S. and Europe) have relatively mild clinical phenotypes, other less common diseases such as Hyper-IgE syndrome or chronic granulomatous disease are associated with significant morbidity and mortality[11, 16-19].

Table 3. Examples of Genetic Disorders that Lead to Neutrophil Dysfunction

Disorder	Gene Defect	Clinical Presentation
Bone Marrow Egress		
WHIM (warts, hypogammaglobulinemia, infections, myelokathexis)	CXCR4	**Peripheral neutropenia**, B-cell lymphopenia, reduced immunoglobulins, infections (HPV)
Adhesion		
LAD (leukocyte adhesion deficiency)	CD18; KINDLIN3, Rac2	**Leukocytosis**, infections, delayed umbilical separation, poor wound healing
Microorganism Killing		
CGD (chronic granulomatous disease)	Components of NADPH Oxidase	**Normal neutrophil count**, infections, inflammatory conditions (IBD)
Myeloperoxidase deficiency	MPO	Mild predisposition to infection (usually with *Candida* spp.)
Other		
Hyper-IgE Syndrome	STAT3	Infections (recurrent skin abscesses, pneumonia), bone fractures, eczema, ↑ **IgE, eosinophilia**

Chronic Granulomatous Disease (CGD)

CGD is a rare disease with an estimated prevalence of approximately 1:200,000[9, 11], caused by mutations in components of the NADPH oxidase complex that predispose affected individuals to a variety of recurrent, life-threatening infections. In addition to infections, CGD patients suffer from the sequelae of dysregulated inflammatory responses (e.g. exuberant granuloma formation)[11, 20-23]. Neutrophils from CGD patients are unable to create reactive oxygen species, and thus lack one of the core innate immune functions of the phagocyte. CGD is predominantly a disease of males (it is X-linked in approximately 70% of cases) and X-linked cases are due to mutations in $gp91^{phox}$. The remaining mutations are transmitted in an autosomal recessive fashion and have been described for $p47^{phox}$ (most common AR form), $p67^{phox}$, $p22^{phox}$, and a recent case of a $p40^{phox}$ mutation [24]. CGD patients suffer a variety of clinical infections with pneumonias being the most common presentation followed by adenitis (Table 4).

Table 4. Common Clinical Presentations of Chronic Granulomatous Disease

Clinical Presentation	% of Patients Affected
Pneumonia	79
Adenitis	53
Liver Abscess	27
Osteomyelitis	25
Lung Abscess	16
Perirectal Abscess	15
Brain Abscess	3
Inflammatory bowel disease	17
Lupus-like syndrome	3
ITP	1

*From a study of 368 CGD patients in the U.S.[11]

Although 76% of patients are diagnosed before the age of 5, approximately 15% of patients presented after the age of 10[11]. Patients with autosomal recessive forms of CGD tend to present later in life, most likely due to their comparatively milder course compared with X-linked patients, resulting in decreased mortality. In the largest CGD cohort study to date, 429 patients from across Europe were followed, and the mean survival time was 37.8 years in the X-linked patients compared to 49.6 years in the autosomal recessive patients[25]. The average age at diagnosis and genetic breakdown were similar to the U.S. study.

Surprisingly, despite having a distinct defect in the innate immune system, only a narrow spectrum of pathogens causes the clinical infections seen in CGD. Importantly, these pathogens, some of which are virtually pathognomonic for CGD, can serve as the "canary" alerting the physician to a possible immune defect. Some pathogens are virtually pathognomonic for CGD. Organisms such as *Chromobacterium violaceum* [26], *Francisella philomiragia* [27] and *Granulibacter bethesdensis* [28-31] should automatically trigger an evaluation for CGD. *G. bethesdensis*, a recently discovered genus and species in the family *Acetobacteraceae*, causes a syndrome of necrotizing lymphadenitis that can be chronic in nature. This pathogen is difficult to isolate from diseased tissue samples and requires sequencing approaches for confirmation. In addition, it is extensively antibiotic resistant and difficult to treat. Given that previous studies fail to find an organism in 40 % of lymphadenitis cases[11], there is ongoing interest in whether there is an under appreciation of this pathogen in the CGD population. As is typical of the pathogens that infect CGD patients, neutrophils from these patients

are unable to kill *Granulibacter* [32]. In the large U.S. study, frequently isolated pathogens were: *Aspergillus* spp, *Staphylococcus aureus*, *Burkholderia cepacia* complex (Bcc), *Nocardia* spp. and *Serratia* spp. Although these pathogens can be seen in other non-CGD hosts, there are usually particular co-morbidities or conditions that are classically associated with the pathogen. For example, the Bcc comprise at least 17 phenotypically similar but genetically distinct species [33-37]. These are environmental organisms [38] and although nosocomial outbreaks from Bcc have occurred, there are really only two hosts that get invasive disease, those with CGD and those with cystic fibrosis (CF)[39-41]. However, the presentation of Bcc lung disease in the CF patient is different than in the CGD patient. Whereas CF patients frequently have endobronchial disease allowing Bcc to be isolated from sputum samples, CGD patients present with focal pneumonia requiring percutaneous biopsy to make a diagnosis[33]. Other CGD pathogens, such as *Staphylococcus aureus*, are seen frequently in many other hosts. Although repeated staphylococcal infections, such as skin abscesses may not automatically trigger an evaluation for CGD, certain presentations should warrant further investigation. This would include spontaneous staphylococcal liver abscess in the absence of other endovascular foci, as this presentation is highly predictive of having CGD [11, 25, 42-44].

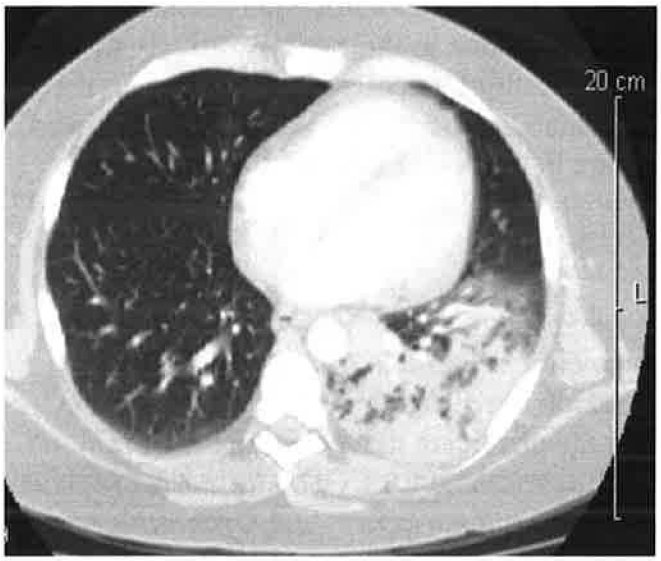


Figure 3. CT scan of the chest showing consolidation in a 32-year-old female. Percutaneous biopsy revealed necrotizing granulomas and cultures grew *Burkholderia cepacia* complex.

Diagnosis of CGD is normally made by documenting that the neutrophils of the individual are incapable of generating superoxide. This is frequently done with either the nitroblue tetrazolium dye reduction test (NBT) or a flow cytometry based assay looking at dihydrorhodamine oxidation (DHR)[45]. DHR utilizes the dye dihydrorhodamine 123 to measure the neutrophil's ability to generate a

respiratory burst when stimulated (Figure 4). This test can have the added advantage of distinguishing X-linked from AR disease.

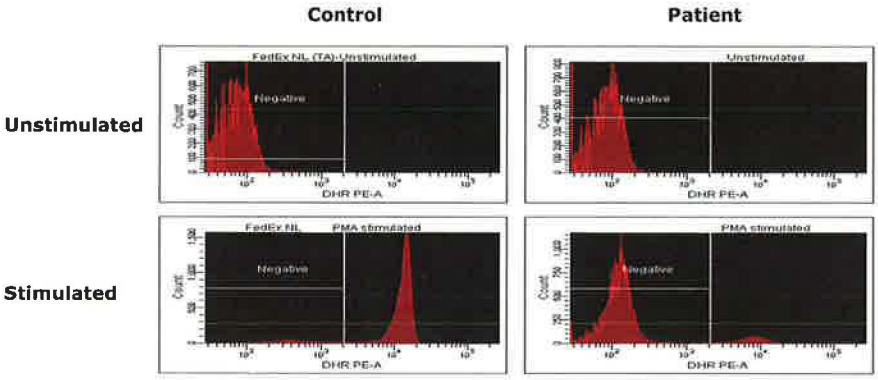
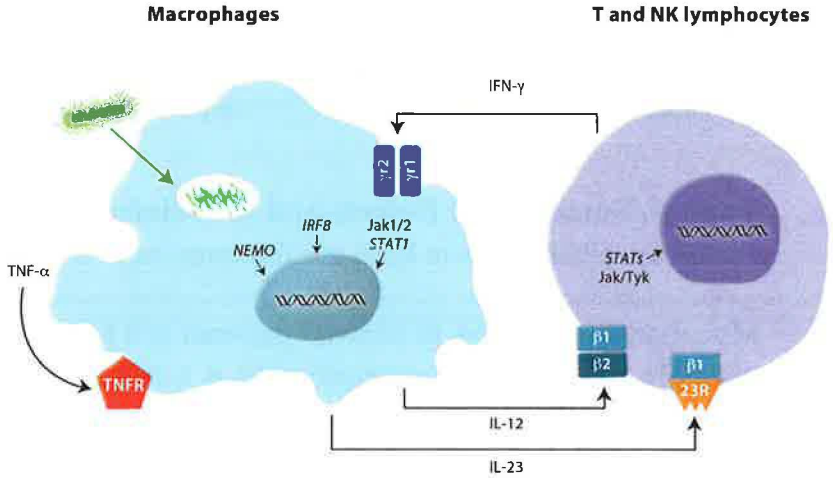


Figure 4. Example of the DHR assay in a patient with *Burkholderia cepacia* infection and suspected CGD. Upon stimulation with PMA (phorbol 12-myristate 13-acetate), control PMNs generate a respiratory burst, resulting in increased fluorescence (rightward shift), while fluorescence remains nearly unchanged in CGD patient PMNs, compatible with an impaired respiratory burst.

Normal Macrophage Function

The host defense against intracellular pathogens depends on the effective interactions between T cells and the other major phagocytic cell, the macrophage. A macrophage that phagocytoses a potential pathogen triggers a series of events that allow for the critical interactions between these cells and thus an effective cell-mediated immune (CMI) response. This circuit relies on critical cytokines such as IL-12 and IFN-γ. IL-12 produced by the macrophage binds to its receptor on T or NK cells that in turn stimulate the production of IFN-γ. IFN-γ then binds to its receptor on the infected macrophage where through downstream signaling molecules it stimulates an antimicrobial response. In addition, cytokines such as TNF-α also play an important role in this process (Figure 4)[10, 46].

Figure 4. Schematic representation of the INF-γ/IL-12 circuit between macrophages and T/NK cells.



Disorders of Macrophage Function

Genetic defects in many of the components of this circuit have been described, with a spectrum of inheritance including X-linked, autosomal recessive and autosomal dominant. These include defects in the IFN-γ receptor components (IFNγR1 and IFNγR2), IL-12β, IL-12Rβ1, and in cellular signaling molecules such as STAT1, TYK2 and NEMO[47]. Mutations in any of these genes result in increased susceptibility to infection with non-tuberculous mycobacteria (NTM); opportunistic pathogens that normally do not cause severe disease in immunocompetent hosts (Table 5). These disorders have been referred to collectively as the Mendelian Susceptibility to Mycobacterial Disease (MSMD). The severity of the clinical presentation in MSMD is highly variable, ranging from local infections to life threatening disseminated mycobacterial disease. In addition (despite the name) there are a variety of other bacterial, viral and fungal pathogens seen in patients with the MSMD defects, thus it really denotes Mendelian susceptibility to a variety of intracellular infections where functional IFN-γ signaling is critical.

Genetic Defect	Associated Pathogens
IL12p40	***M. bovis* BCG, non-tuberculous *mycobacteria* spp.**, *M. tuberculosis*, **Salmonella spp.**, *Nocardia*
IL12Rβ1	***M. bovis* BCG, non-tuberculous *mycobacteria* spp.**, *M. tuberculosis*, **Salmonella spp.**, *Paracoccidioides brasiliensis, Leishmania*
IFNγR1	***M. bovis* BCG, non-tuberculous *mycobacteria* spp.**
IFNγR2	***M. bovis* BCG, non-tuberculous *mycobacteria* spp.**
STAT1 (signal transducer and activator of transcription-1)	***M. bovis* BCG, non-tuberculous *mycobacteria* spp., *Salmonella* spp., viruses (HSV, EBV), chronic mucocutaneous candidiasis, *coccidioides immitis, histoplasma capsulatum***
NEMO (nuclear factor-κβ-essential modulator)	***Streptococci* spp., non-tuberculous *mycobacteria* spp.**, *H. influenzae, S. aureus*

Table 5. Summary of Defects and Associated Pathogens in the IFNγ/IL-12 Pathway. (Pathogens in bold are the ones most frequently seen)

Mycobacterial disease in MSMD patients can present in a variety of ways. For example, mycobacterial osteomyelitis is a common presentation in patients with

IFNγR1 mutations[48]. Because BCG vaccine is used in many areas of the world, one of the first clinical presentations in these patients will be abscess formation, lymphadenitis and osteomyelitis at some point after receiving the vaccine. In one Japanese study, BCG was the most common pathogen found (seen in 82.6% of patients)[49]. Currently, the most common genetic cause of MSMD is IL-12Rβ1 deficiency, which was discovered in 1998[50]. In a large study of IL-12Rβ1-deficient patients from 30 countries, mycobacteria were the most frequent cause of infections (109/132 patients or 83%)[51]. Out of the 108 patients that were vaccinated with BCG, 84 developed BCG-related disease. Environmental mycobacteria were seen in 21/132 patients with the most common being *M. avium*. In addition, other pathogens that have been seen include: *Salmonella* spp., *Nocardia* spp., and various fungi such as *Coccidioides immitis* [52, 53], *Histoplasma capsulatum*, mucocutaneous candidiasis, and *Paracoccidioides*.

As is seen with neutrophil defects, the pathogens seen in disorders of macrophage dysfunction should trigger an alarm in the physician's mind about a possible underlying phagocyte disorder. Histoplasmosis is a good example of an opportunistic disease that rarely occurs despite broad exposure to the etiologic agent. *Histoplasma capsulatum* is a soil dwelling dimorphic fungus and is reported worldwide. It is endemic in the Americas as well as parts of Africa and Europe[54]. There is a broad spectrum of clinical manifestations that range from an asymptomatic infection to a brief self-limited illness to progressive disseminated disease. Underlying co-morbidities that are associated with disseminated disease include immunosuppression from AIDS, malignancy, hematologic or solid-organ transplant, chronic steroid use, and treatment with TNFα inhibitors[55-57]. In the absence of these secondary conditions, a patient with disseminated histoplasmosis should be evaluated for a defect in the IFNγ/IL-12 pathway.

STAT 1 Deficiency

STAT 1 (Signal Transducer and Activator of Transcription) is a critical cytosolic protein important in INFγ signaling. After INFγ binds to its receptor, STAT is phosphorylated and forms a dimer that translocates to the nucleus and up-regulates INFγ related genes. The first defect in STAT1 was discovered in 2001 in a patient with mycobacterial infection[58]. Since that time, a variety of different mutations have been found that have different proposed effects on gene function as well as different infectious manifestations (Figure 5,6)[58-61].

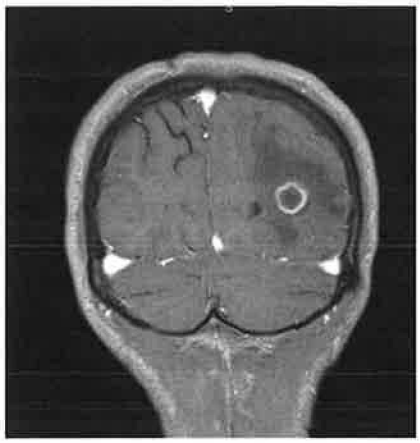
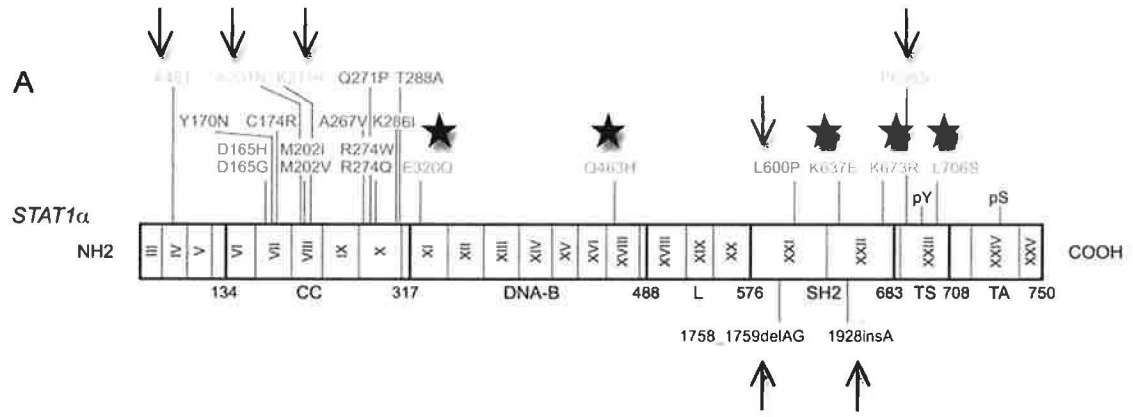


Figure 5. MRI of a patient with histoplasmosis of the brain and a STAT1 mutation.

Figure 5. Diagram of the STAT1 gene with known mutations and pathogens.



The arrows denote mutations that were autosomal recessive, resulting in partial or complete STAT1 deficiency and suffer from disseminated intracellular bacterial infections with *Salmonella*, *Mycobacteria bovis* (BCG) and/or viral disease including HSV and RSV. The stars denote mutations that are autosomal dominant and cause partial STAT1 deficiency. These patients also suffer from mycobacterial infections, both with BCG and environmental mycobacteria. Finally the other mutations listed around the CC (coiled-coiled) domain are autosomal dominant and are associated with gain-of-function of STAT1. These patients develop infections with chronic mucocutaneous candidiasis as described by Liu et al.[59]. However, patients with similar mutations have been found who present with other disseminated fungal infections, including histoplasmosis and coccidiomycosis (Sampaio EP et al.; manuscript in review). This adds to the spectrum of pathogens that are seen in STAT1 genetic defects.

Conclusions

There are a variety of phagocyte defects that while rare individually, in the aggregate reach numbers that adult physicians are likely to encounter during the course of their career. In addition, therapeutic advances make the possibility of delayed diagnosis more likely, pushing off the discovery of some of these defects into adulthood. The pathogen that a patient presents with can yield important clues as to the innate immune pathway that may be dysfunctional and should warrant a closer immunologic evaluation.

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