Immune dysfunction in Niemann-Pick disease type C

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Abstract
Lysosomal storage diseases are inherited monogenic disorders in which lysosome function is compromised. Although individually very rare, they occur at a collective frequency of approximately one in five thousand live births and usually have catastrophic consequences for health. The lysosomal storage diseases Niemann-Pick disease type C (NPC) is caused by mutations predominantly in the lysosomal integral membrane protein NPC1 and clinically presents as a progressive neurodegenerative disorder. In this article we review data that demonstrate significant dysregulation of innate immunity in NPC, which occurs both in peripheral organs and the CNS. In particular pro-inflammatory responses promote disease progression and anti-inflammatory drugs provide benefit in animal models of the disease and are an attractive target for clinical intervention in this disorder.

Keywords: cytokine, inflammation, lysosomal storage disease, lysosome, microglia, neurodegeneration, Niemann Pick type C.

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Lysosomal storage diseases
Lysosomal storage diseases (LSDs) are a group of metabolic disorders that result from inherited defects in proteins required for normal lysosome function (Ballabio and Gieselmann 2009; Schultz et al. 2011). Affected proteins can include catabolic enzymes, integral membrane proteins, and proteins required for generating specific post-translational modifications of proteins (Futerman and van Meer 2004; Platt et al. 2012). To date, approximately 70 discrete LSDs have been described that are individually rare; however, collectively they occur at a prevalence of 1:5000 live births, which may be an underestimate owing to cases being either undiagnosed or misdiagnosed (Cox and Cachon-Gonzalez 2012).

There are two striking features of LSDs. The first is that disturbance of lysosomal function frequently has severe consequences for the function of cells, organ systems, and the body as a whole, leading to premature death (Platt et al. 2012; Platt 2014). This is perhaps not surprising in light of the accumulating evidence that the lysosome is involved in far more than macromolecule catabolism and re-cycling. The lysosome is now recognized to be an organelle that has a much broader range of functions including signaling, secretion, and regulation of energy metabolism (Settembre et al. 2013). Second, distinct LSDs that result from the inactivation of different lysosomal proteins often share similar pathologies (Vitner et al. 2010). For example, inappropriate activation of the innate immune system in the form of inflammation is especially frequent in LSDs, but intriguingly it remains an open question as to how exactly lysosomal storage triggers pro-inflammatory pathways (Platt 2014).

Niemann-Pick disease type C
Niemann-Pick disease type C (NPC) is an autosomal recessive condition caused by mutations in either of two independent genes. Mutations in NPC1, an integral transmembrane protein of the limiting membrane of the lysosome (Higgins et al. 1999) accounts for ~95% of all clinical cases whereas mutations in NPC2, a soluble cholesterol binding protein are responsible for the remainder (Platt et al. 2014). Therefore, NPC is unlike the majority of LSDs, which are caused by mutations in single genes that encode a lysosomal hydrolase. Patients with mutations in either NPC1 or NPC2 are phenotypically indistinguishable and because loss of activity of either protein is not compensated for by the presence of the other protein, it is probable that they function in a common cell...
biological pathway (Dixit et al. 2011). For the purpose of this review our discussion will concentrate on the consequences of impaired NPC1 function, as most information is available on NPC1 disease. Clinically, affected individuals are usually diagnosed in childhood although adult onset variants also occur and are under-diagnosed (Wassif et al. 2015). The course of disease is dominated by progressive neurodegeneration, which presents as cerebellar ataxia, dysphagia, dementia and premature death, typically within the second decade of life (Vanier 2010). Almost three hundred different mutations in NPC1 have been identified to date, but as yet there is neither a clear structure–function relationship that has been established, nor a functional assay for NPC1 (Millat et al. 1999; Ribeiro et al. 2001).

The phenotype of NPC1-deficient cells

Probably the most prominent feature of NPC cells is the accumulation of free cholesterol in the late endosome/lysosome (Fig. 1). Although this remains the basis for the clinical diagnostic test (Vanier 2010) it is contentious whether it is disease causing or a secondary consequence of the disease. In fact a complex array of lipid species are stored in NPC including glycosphingolipids (GSLs), sphingomyelin, and sphingosine, the latter generated from ceramide catabolism (Rosenbaum and Maxfield 2011). Because storage does not occur as a direct result of impaired catabolic activity NPC is classified as secondary LSD. The function of NPC1 is itself currently unresolved and although the prevailing view is that it is involved in removing cholesterol from the lysosome, evidence that the purified protein can bind the sterol may indicate its requirement as a co-factor rather than support the hypothesis that NPC1 is responsible for moving cholesterol out of the acidic compartment. Further support for this has been the demonstration that cholesterol can move effectively from the lysosome to mitochondria in NPC1 cells (Kennedy et al. 2014). A combination of experimental data reinforces an alternative model of the pathogenic cascade (Fig. 2). NPC1 has greatest homology with the resistance-nodulation-division family of prokaryotic permeases and has been demonstrated to have activity as an efflux pump (Davies et al. 2000). When NPC1 is inactivated in healthy cells the first metabolite to accumulate is sphingosine (which also builds up in tissues in patients and animal models, Rodriguez-Lafrasse et al. 1994), which requires active transportation out of the lysosome because it is protonated at acidic pH (Lloyd-Evans et al. 2008). A consequence of the storage of sphingosine is inhibition of the filling of the acidic compartment with calcium. This could be indirect via protein kinase C inhibition (Walter et al. 2009) or a direct effect of sphingosine on the protein that fills the acidic store with calcium, the identity of which remains unknown. Whilst the precise biological roles of calcium release from the lysosome are not fully defined it is known that fusion and trafficking in the endocytic pathway are uniquely dependent upon calcium mobilization from the lysosome (Morgan et al. 2011). Therefore, impaired filling of the lysosome with the cation, as a result of sphingosine accumulation, means insufficient calcium can be released, which in turn leads to a block in endosome/lysosome trafficking and fusion. This is then the cause of the secondary storage of cholesterol, GSLs, and sphingomyelin (Fig. 1). All of these defects, including sphingosine accumulation, the lysosomal calcium deficit, impaired calcium release, and prevention of normal endocytic trafficking have been confirmed independently in multiple studies in NPC cells (Rodriguez-Lafrasse et al. 1994) (Chen et al. 2010) (Shen et al. 2012; Tamari et al. 2013).

NPC and innate immune responses

Because neurodegeneration is the main clinical feature of NPC disease, it is unsurprising that a major research focus has been on the effects of loss of NPC1 activity within the CNS. However, because NPC1 is expressed in all cells, its loss is very likely to have widespread consequences. In this review we will discuss the evidence that mutations in NPC1 impact significantly on multiple tissues and cells, in partic-
ular within the immune system and result in inflammation and altered innate immune responses.

**Animal model of NPC; the Npc1<sup>−/−</sup> (BALB/cNctr-Npc1<sup>m1N/J</sup>) mouse**

Our understanding of NPC has been advanced enormously by studies of an authentic mouse model, the Npc1<sup>−/−</sup> (BALB/cNctr-Npc1<sup>m1N/J</sup>) mouse, which has almost total absence of the protein and displays all the hallmarks of the clinical disease (Loftus et al. 1997). This mutant strain arose spontaneously and has a lifespan in the range of 10–14 weeks and therefore has a course of disease more acute that the vast majority of patients. The mutant mouse has been exploited successfully not only for determining the ontogeny of disease and underlying pathogenic mechanisms but also for the evaluation of experimental therapies. Analyses using these mice have been undertaken at the whole animal, cellular, and molecular levels (Baudry et al. 2003; Smith et al. 2009; Cologna et al. 2012, 2014).

Prior to about 4–5 weeks of age Npc1<sup>−/−</sup> mice have no discernible behavioral indication of disease that distinguishes them from wild-type littermates. First indications of behavioral deficits, such as tremor and ataxic gait, appear by weeks 5–6; by weeks 7–8 defects in motor coordination become more apparent, and by 9–10 weeks ataxia is advanced and accompanied by increased loss in weight and poor coat condition as feeding and drinking becomes difficult (humane end point applied) (Fig. 3) (Smith et al. 2009). Although the BALB/cNctr-Npc1<sup>m1N/J</sup> strain has been the most intensively studied, there are also other authentic mammalian animal models of NPC. Interestingly, loss of Npc1 in mice on the C57BL/6 genetic background results in more acute disease relative to the BALB/cNctr-Npc1<sup>m1N/J</sup> mouse (Parra et al. 2011), suggestive of genetic modifiers. A feline model of NPC has also been characterized (Brown et al. 1994) and successfully used to trial experimental therapies (Stein et al. 2012).

**Neuroinflammation and neurodegeneration in NPC**

Pathology within the CNS is a feature of most LSDs, probably because neurons are uniquely vulnerable to perturbation of normal lysosomal activity (Jmoudiak and Futerman 2005; Bellettato and Scarpa 2010; Vitner et al. 2010). Activation of the innate immune system is also a very
common feature of neurodegenerative LSDs (Jeyakumar et al. 2003) (Bellettato and Scarpa 2010; Vitner et al. 2010).

Interestingly, microglial activation (as indicated by up-regulation of CD68 expression) and astrogliosis (Pekny and Nilsson 2005) are apparent from as early as 2 and 4 weeks of age respectively in Npc1−/− mice (Baudry et al. 2003), prior to any overt behavioral symptoms. Whilst NPC-activated microgla in GM1 and GM2 gangliosidoses mice (Jeyakumar et al. 2003), they do not become positive for MHC class II expression, indicating a different activation state, the significance of which is not currently understood (Smith et al. 2009). Neuropathology in NPC develops in a distinct temporal and spatial pattern, with cell loss in both motor and sensory pathways, in addition to the characteristic progressive death of Purkinje cells in the cerebellum (Pressey et al. 2012). Because Npc1 activity is absent throughout the CNS of BALB/cNctr-Npc1m1/J mice an important question has been to what extent does deletion in particular cell types drive disease. Although data points toward cell autonomous neurodegeneration (Lopez and Scott 2013), non-cell autonomous mechanisms cannot be absolutely excluded because of the complex interdependence of different cell populations within the CNS. For example, the lifespan of Npc1−/− mice was extended threefold by expression of functional Npc1 in astrocytes (Zhang et al. 2008), greater than fivefold when expressed in neurons and when combined there was an additive effect, but disease symptoms in motor systems still occurred, albeit significantly delayed (Borbòn et al. 2012).

Changes in gene expression in the CNS that underlie the pathologic cascade have also been documented and one pathway that has been identified is a network of pro-inflammatory genes (Cologna et al. 2014). Comparable studies performed on post-mortem patient cerebellum with those from the mutant mouse revealed transcriptional changes and identified complement C3 as the only gene found to be elevated in all samples analyzed (Cologna et al. 2014).

It is still not understood why brain inflammation is triggered in NPC and other LSDs. Clearly, neuroinflammation as a generalized response is independent of the molecular identity of the storage material, because it is universal amongst neurodegenerative LSDs. However, the precise nature of the inflammatory profile is very likely to differ between specific LSDs and is very probably influenced by both the chemical composition of the storage and the underlying mechanistic defect. It is recognized that inflammation is a response to tissue damage or stress and is a protective mechanism that can result in induction of appropriate repair processes (Chovatiya and Medzhitov 2014). However, in chronic conditions the failure of inflammation to resolve, presumably because the pro-inflammatory stimulus remains, means the destructive phases of the response are significantly extended.

Microglia, the resident myeloid cell populations of the brain are considered to be the most important source of pro-inflammatory molecules, including cytokines, chemokines, and reactive oxygen species within the CNS (Gonzalez-Scarano and Baltuch 1999) (Ransohoff and Perry 2009) as well as providing protective mechanisms (Griffiths et al. 2009; Napoli and Neumann 2010). Several scenarios can be envisaged to explain why microglia are activated in LSDs. It may be as a result of accumulation of storage materials in microglia themselves affecting signaling pathways. On the other hand, as sentinels within the CNS it may be microglial detection of storage in other cell populations, particularly neurons, that is the trigger. Of course, it may not be actual storage, but rather a loss of cellular homeostasis beyond the normal range. Loss of neurons, especially by necrotic death is also very likely to provoke innate immune-mediated inflammation (Kono and Rock 2008). An important activity of microglia is the phagocytosis of apoptotic cell debris (Napoli and Neumann 2009), but it is not known if this scavenging activity is normal or impaired in NPC and if is the latter, whether it also contributes to the inflammatory profile (Palin et al. 2008). Unfortunately, at this time we have an incomplete understanding of the temporal and spatial details of inflammation and neurodegeneration in NPC.

One of the most important issues that have been resolved is the question of whether neuroinflammation actively promotes pathogenesis. The ability of anti-inflammatory therapies, such as non-steroidal drugs (NSAIDs), aspirin and ibuprofen to significantly extend the lifespan, protect against microglial activation and Purkinje cell loss and delay symptom onset in Npc1−/− mice (Smith et al. 2009) confirmed that neuroinflammation enhances disease and when used in combination with agents that inhibit GSL biosynthesis and modulate calcium they can maximize therapeutic benefit (Williams et al. 2014).

### Deficits in the NPC peripheral immune system

**Natural killer cells**

The immune system in peripheral tissue and the CNS are intimately connected at a number of levels and so the immune system as a whole merits study in these diseases. Natural killer (NK) cells are an immune cell population that, as an effector sub-group of lymphocyte they express a number of inflammatory receptors, which engage ligands on target cells and when appropriate release cytotoxic products via degranulation (Lanier 2008).

The first lipid to accumulate in the lysosome when NPC1 is inactivated is sphingosine. In healthy cells sphingosine has two potential fates; it can either be recycled back to form ceramide for reutilization in the salvage pathway of GSL.
biosynthesis or phosphorylated by two specific kinases to yield sphingosine-1-phosphate (S1P), a bioactive lipid that has multiple known functions (Maceyka and Spiegel 2014). During inflammation immune cells enter and leave sites of tissue damage by the process of regulated cell migration and trafficking (Butcher and Picker 1996). S1P levels differ significantly between the blood, lymph, and tissues (higher in the first two; lower in the latter) and this gradient has a major role in affecting lymphocyte trafficking as it forms a chemotactic gradient (Cyster and Schwab 2012). Speak Speak et al. (2014) hypothesized that the accumulation of sphingosine in the lysosome in NPC would mean reduced generation of S1P (phosphorylation of sphingosine occurs outside of the lysosome) and result in alteration of the normal systemic/tissue gradient and thereby affect NK cell trafficking. Firstly, the authors confirmed a 10-fold increase in the sphingosine content of lymph nodes in Npc1−/− mice and a proportional decrease in conversion to S1P (only a three-fold increase), suggesting the likelihood of a defect in the gradient between lymphatic fluid and node. Second, as predicted there was a significant increase in the frequency of NK cells in multiple organs as compared to control animals and a corresponding decrease in the circulation. The distribution of NK cells in Npc1−/− mice is similar to that reported in animals deficient in a specific S1P receptor (Walzer et al. 2007). Comparable differences in frequencies were also measured in blood from NPC patients and interestingly, carrier genotypes also failed to show an age-related expansion in NK cell numbers. In addition, analysis confirmed an alteration in NK cell development and phenotype (maturation status) as well as frequency in Npc1−/− animals and patients. Functionally, deficient cells in the mouse were significantly compromised in their ability to kill target cells (cytotoxicity), which correlated with impaired degranulation and reduced calcium release from the lysosome (Speak et al. 2014). These data strongly implicate the requirement for lysosomal calcium in cytotoxic granule (specialized lysosome-related organelles) secretion by NK cells, which has also been demonstrated for cytotoxic T-cells degranulation if acidic store calcium release is impaired (Davis et al. 2012). Furthermore, a prediction from this would be that other Npc1−/− cell types might also have diminished secretion of lysosome-related organelles.

**Invariant natural killer T cells and LSDs**

Invariant natural killer T cells (iNKT) cells are a specialized subset of T lymphocytes that bear an invariant TCRα chain and express markers shared with NK cells (Bendelac et al. 2007). They are key effector cells in innate immunity and modulate the activities of the adaptive immune system, especially in host defense against pathogens and cancer (Brennan et al. 2013). Unlike conventional T cells, which respond to antigen presenting cells that express peptides in the context of MHC molecules, iNKTs are activated by GSL ligands presented by the MHC-related molecule CD1 (CD1a-e in humans; CD1d in the mouse) (Speak et al. 2008). Importantly, both positive selection of iNKT cells in the thymus and their maintenance in peripheral organs is dependent upon presentation of specific endogenous lipid ligands by CD1 (Mendiratta et al. 1997; Gapin et al. 2001). Because loading of CD1 with lipid molecules occurs in late endocytic/lysosome compartments in the mouse, diseases that alter the distribution, nature or abundance of GSLs (such as LSDs) have the potential to render this process ineffective and thereby affect the frequency and properties of iNKT cells. Consistent with this, it was found that multiple mouse models of LSDs (that store diverse GSL species), including NPC, had decreased numbers of iNKT cells and their maturation was compromised (but T cells specific for peptide ligands were not affected) (Gadola et al. 2006). GSL-triggered cytokine release was significantly reduced and presentation of endogenous lipids was impaired in all models, but interestingly only Npc1−/− mice had lower cell surface expression of CD1d (Gadola et al. 2006). However, in contrast to the situation in the mouse, analysis of NPC patients revealed normal frequencies and functions of iNKT cells (Speak et al. 2012). This difference is most likely due to differences in trafficking requirements for iNKT cells between the two species. The evidence is that lipid loading occurs in the early endosome in humans, not in the late endocytic system as it is in the mouse, so is therefore not affected in NPC patients (Chen et al. 2007).

**Conclusions**

In this review we have highlighted that LSDs are not just diseases of CNS dysfunction but also involve activation of the innate immune system, leading to chronic inflammation that actively contributes to the disease. There are multiple routes of communication between the peripheral immune system and the CNS, including both direct ones mediated by neural circuits (Olofsson et al. 2012) and systemic mechanisms, in the form of circulating cytokines, acute phase proteins and other pro-inflammatory molecules (Allan and Rothwell 2001). It is well established that the ongoing neurodegenerative processes in the CNS are exacerbated by induction of inflammation at peripheral sites (Perry et al. 2003). Also, neuropathology frequently results in the recruitment of immune cells from peripheral sites into the CNS and should their generation or functioning be ineffective, any neuroprotective benefit their mobilization may offer will be lost. A particularly important reason for exploring the nature of the altered innate immune responses in LSDs is that the vast majority of these disorders are currently without effective treatment and anti-inflammatories have shown benefit in relevant models and are attractive as adjunct
therapeutics. Therefore, targeting the immune system merits clinical evaluation.

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