Thyroid hormone metabolism in innate immune cells

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Abstract

Thyroid hormone (TH) metabolism and thyroid status have been linked to various aspects of the immune response. There is extensive literature available on the effects of thyroid hormone on innate immune cells. However, only recently have authors begun to study the mechanisms behind these effects and the role of intracellular TH metabolism in innate immune cell function during inflammation. This review provides an overview of the molecular machinery of intracellular TH metabolism present in neutrophils, macrophages and dendritic cells and the role and effects of intracellular TH metabolism in these cells. Circulating TH levels have a profound effect on neutrophil, macrophage and dendritic cell function. In general, increased TH levels result in an amplification of the pro-inflammatory response of these cells. The mechanisms behind these effects include both genomic and non-genomic effects of TH. Besides a pro-inflammatory effect induced by extracellular TH, the cellular response to pro-inflammatory stimuli appears to be dependent on functional intracellular TH metabolism. This is illustrated by the fact that the deiodinase enzymes and in some cell types also thyroid hormone receptors appear to be crucial for adequate innate immune cell function. This overview of the literature suggests that TH metabolism plays an important role in the host defence against infection through the modulation of innate immune cell function.

Key Words
- thyroid hormone metabolism
- innate immune cells
- deiodinases
- thyroid hormone receptors

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Introduction

The interplay between the endocrine and immune system is well established (Besedovsky & del Rey 1996, Klein 2006, Schaefer & Klein 2011). Thyroid hormone (TH) metabolism and TH status have been linked to various aspects of the immune response (Boutzios & Kaltsas 2000, Klein 2006, De Vito *et al*. 2011), and there is an extensive body of literature available on the effects of TH on various types of innate immune cells (De Vito *et al*. 2011). However, very few of these studies analyse the mechanisms behind the effects of TH or the role of intracellular TH metabolism in innate immune cells. In recent years, the role of TH metabolism in innate immune cell function has been studied in more detail, and it has been suggested that innate immune cells are important T_3 target cells and that intracellular TH plays an essential role in the function of several cell types of the innate immune system. This review provides an overview of the elements of intracellular TH metabolism present in innate immune cells and the role and effects of intracellular TH metabolism in these cells. It focuses specifically on the phagocytic innate immune cells: neutrophils, macrophages and dendritic cells.

Thyroid hormone production and metabolism

The regulation of plasma TH levels is conducted via a classic endocrine negative feedback loop involving

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the hypothalamic–pituitary–thyroid (HPT) axis. Hypophysiotropic neurons within the paraventricular nucleus of the hypothalamus produce thyrotropin-releasing hormone (TRH), which in turn stimulates the thyrotroph cells of the anterior pituitary to synthesize and secrete thyroid-stimulating hormone (TSH) (Harris *et al.* 1978). TSH then stimulates the thyroid gland to produce thyroid hormones in the form of thyroxine (T_4) and triiodothyronine (T_3) (Miot *et al.* 2015). These hormones are secreted into the circulation, and their plasma levels in turn regulate the hypothalamic release of TRH, completing the feedback loop.

The thyroid gland mainly produces T_4, which functions as a prohormone and requires conversion to T_3 to become biologically active, although a minor role for direct actions of T_4 has also been reported (Davis *et al.* 2016). This conversion occurs at the cellular and tissue level, enabling the local regulation of TH bioavailability.

Intracellular thyroid hormone metabolism

TH is actively transported into the cell by TH transporters. There are several families of TH transporters including organic anion transporter polypeptides (OATP), monocarboxylate transporters (MCT) and large neutral amino acid transporters (LAT) (Bernal *et al.* 2015, Visser 2016). Of these transporters, MCT8 is the only one to transport TH exclusively. The other transporters are also capable of transporting additional substances including steroids and amino acids (Bernal *et al.* 2015). Studies on transgenic mouse models and observations in patients with pathogenic mutations in TH transporters indicate that MCT8, MCT10 and OATP1C1 are the main transporters of (patho)physiological importance to TH transport *in vivo* (Bernal *et al.* 2015, Visser 2016). MCT8 preferentially transports T_4, whereas MCT10 preferentially transports T_3 (Visser 2016). OATP1C1 transports T_3, T_4 and rT_3 with high specificity; however, it has the lowest affinity for T_4 out of these transporters (Pizzagalli *et al.* 2002, Bernal *et al.* 2015, Visser 2016). Transporter expression is cell type-specific and differences in distribution have been observed between humans and rodents (Bernal *et al.* 2015).

After being transported into the cell, TH is metabolized by the iodothyronine deiodinases. This a family of enzymes that remove an iodine atom from the phenolic or tyrosyl ring of TH (Bianco & Kim 2006, Visser & Peeters 2012). Type 1 deiodinase (D1) is capable of both inner and outer ring deiodination. Although it has a lower affinity for T_4 than the other deiodinases, it is highly expressed in the liver where it is thought to be the main source of local T_3 and to be important for the clearance of rT_3 (Bianco & Kim 2006). Type 2 deiodinase (D2) is capable of phenolic or outer ring deiodination resulting in the conversion of the prohormone T_4 to the active hormone T_3 (Bianco & Kim 2006). Approximately 80% of extra-thyroidal T_3 is derived from peripheral deiodination of T_4, mainly by D1 in the liver and D2 in skeletal muscle (Visser & Peeters 2012). Type 3 deiodinase (D3) is an inner ring deiodinase that converts T_4 and T_3 to their respective inactive metabolites rT_3 and T_2 (Bianco & Kim 2006).

Besides deiodination, there are other minor pathways of TH metabolism including sulfation, glucaronidation and ether-linked cleavage. The precise mechanisms of these metabolic pathways are beyond the scope of this review and have been discussed by other authors in more detail (Wu *et al.* 2005, Visser & Peeters 2012).

The classical pathway through which TH exerts its biological effects is by binding to the nuclear TH receptors (TRs). Upon binding of T_3, these TRs are capable of directly initiating or inhibiting gene transcription (Brent 2012, Mullur *et al.* 2014). There are several TR isoforms that are differentially expressed in a tissue- and cell type-specific manner (Brent 2012). The isoforms that are capable of binding T_3 are TRα1, which is widely expressed in cardiac and skeletal muscle, the central nervous system and bone, TRβ1, which is mainly present in the brain, liver and kidney and TRβ2, which is expressed in the hypothalamus and pituitary (Cheng *et al.* 2010, Brent 2012).

There is increasing evidence that THs also act via non-genomic pathways (Davis *et al.* 2016). The pathways involved in non-genomic TH actions are initiated by binding of TH to another receptor than the intracellular TRs, for example to the receptor on plasma membrane integrin αvβ3 (Davis *et al.* 2016). The classic pathways of TH action and the rapid non-genomic pathways activated by TH are not completely independent from each other as rapid non-genomic actions of TH can affect intracellular TRs and even require TRs in certain cell types (Davis *et al.* 2016, Flamant 2016).

Innate immune cells

The innate immune system is responsible for the host defence against invading pathogens. The cells of the innate immune system identify microbes, initiate an inflammatory response and can either directly phagocytose and kill pathogens or recruit other innate

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or adaptive immune cells to the site of infection. Innate immune cells are derived from haematopoietic stem cells in the bone marrow. These cells can be mobilized from the blood or bone marrow upon infection. Alternatively, innate immune cells travel from the bone marrow to the tissue and patrol there for invading pathogens; these are known as tissue-resident cells. This review will focus on the phagocytic innate immune cells, which comprise neutrophils, monocytes/macrophages and dendritic cells.

Neutrophils

Neutrophils are the first cells to be recruited to the site of inflammation and are the most abundant type of blood leukocyte, comprising 50–75% of circulating leukocytes in humans (Borregaard 2010, Kolaczkowska & Kubes 2013, Bardoel *et al*. 2014). Circulating neutrophils are short-lived cells that are generated in the bone marrow by haematopoietic stem cells (Borregaard 2010).

Upon inflammation and infection, neutrophils from the circulation are recruited to the site of inflammation. Inflammatory mediators are recognised by neutrophils, after which they adhere to the vascular endothelium close to the site of infection before transmigrating into the extravascular tissue. Extravasated neutrophils then migrate to the place of inflammation where they can then kill invading pathogens and secrete inflammatory mediators further stimulating the immune response and recruiting other innate and adaptive immune cells (Kolaczkowska & Kubes 2013).

Neutrophils are highly specialized cells that have multiple microbial killing mechanisms at their disposal. These mechanisms have been discussed extensively in other reviews (Borregaard 2010, Kolaczkowska & Kubes 2013, Bardoel *et al*. 2014), therefore, we will only include a brief overview of these processes here. The three main killing mechanisms utilized by neutrophils are degranulation, the production of reactive oxygen species and the generation of neutrophil extracellular traps (Kolaczkowska & Kubes 2013, Bardoel *et al*. 2014). Upon phagocytosis of a pathogen, neutrophils can release various bactericidal elements into the phagosome. Some of these elements are antimicrobial proteins and enzymes that are formed sequentially during neutrophil development and stored in intracellular granules (Borregaard & Cowland 1997, Borregaard 2010). Upon phagocytosis, these granules can fuse with the phagosome or the plasma membrane, releasing their contents in a process known as degranulation (Borregaard 2010). Neutrophils are also capable of generating reactive oxygen species (ROS) in the phagosome using the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. An important extracellular killing mechanism is neutrophil extracellular traps (NETs) (Brinkmann *et al*. 2004). NETs are composed of neutrophil chromatin to which antimicrobial proteins and ROS are bound (Brinkmann *et al*. 2004, Kolaczkowska & Kubes 2013). The release of these NETs enables neutrophils to effectively trap and kill extracellular bacteria, but also eventually results in the death of the neutrophil (Brinkmann *et al*. 2004).

Monocytes and macrophages

Monocytes and macrophages are mononuclear phagocytic cells. Monocytes are continuously generated in the bone marrow by haematopoiesis and released into the circulation where they constitute 10% of circulating human leukocytes. There is also a considerable monocyte reservoir in the spleen and lungs that can be mobilized on demand (Ginhoux & Jung 2014). Circulating monocytes can extravasate to tissues both during the steady state and during inflammation where they can differentiate into macrophages or dendritic cells (Shi & Pamer 2011). An alternative subset of macrophages is the tissue-resident macrophages. Until recently, these were thought to be continuously replenished from the circulating monocyte pool. Tissue-resident macrophages are now known to be derived from embryonic precursors that colonize the tissues prenatally (Mass *et al*. 2016). These cells, which include Kupffer cells and microglia, are also able to maintain their populations in adult tissues due to local cell proliferation independently of circulating monocytes (Hashimoto *et al*. 2013, Ginhoux & Jung 2014, Mass *et al*. 2016). The various tissue-resident macrophages comprise distinct cell populations whose phenotype differs strongly between tissues (Murray & Wynn 2011).

After entering the tissue, macrophages can change their phenotype due to various stimuli, allowing them to adapt to a wide subset of roles. This process is known as polarization. Polarized macrophages are generally classified into M1 or classically activated macrophages, which are pro-inflammatory cells, and M2 or alternatively activated macrophages, which is a heterogeneous group of cells that have a more anti-inflammatory profile (Murray & Wynn 2011). M1 macrophages are important in antimicrobial defence and the recruitment of neutrophils and T cells to the inflamed tissue (Murray & Wynn 2011). They are also capable of antigen presentation and can

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elicit a T-cell response (Hume 2008). M1 polarization is accompanied by changes in cellular metabolism, shifting towards enhanced glycolysis (Freemerman *et al*. 2014, Galvan-Pena & O'Neill 2014, Zhu *et al*. 2015). Essential components of adequate pro-inflammatory macrophage function are phagocytosis, the generation of ROS by NADPH oxidase and the generation of reactive nitrogen species (RNS), which is mediated by inducible nitric oxide synthase (iNOS) (Weiss & Schaible 2015). M2 macrophages are tolerogenic and immunomodulatory cells that are involved in wound healing and tissue remodelling (Murray & Wynn 2011). This is also accompanied by metabolic changes resulting in enhanced fatty acid oxidation and mitochondrial oxidative phosphorylation (Vats *et al*. 2006, Galvan-Pena & O'Neill 2014, Zhu *et al*. 2015). More recent data suggest that macrophage polarization is not as clear cut as these two phenotypes and represents more of a spectrum ranging from pro- to anti-inflammatory (Hume 2015).

Dendritic cells

Dendritic cells are unique innate immune cells that are not only capable of phagocytosing pathogens but also function as antigen-presenting cells. Dendritic cells thus bridge the gap between innate and adaptive immunity and can shape the T-cell response. The dendritic cell (DC) population is derived from the haematopoietic lineage and is more heterogeneous than previously thought. Currently four main types of DCs are recognised: classic DCs, plasmacytoid DCs, Langerhans cells and monocyte-derived DCs. All these subsets derive from a common myeloid progenitor (Satpathy *et al*. 2012, Pearce & Everts 2015). Classic DCs and monocyte-derived DCs are cells specialized in phagocytosis of pathogens. Unstimulated or immature classic DCs have a short half-life and are continuously replenished from precursors in the bone marrow (Satpathy *et al*. 2012). After activation these cells undergo considerable morphological changes and are characterized as mature classic DCs. Just as in macrophages, the activation of DCs is accompanied by a shift in cellular metabolism favouring glycolysis over oxidative phosphorylation (Pearce & Everts 2015). Mature DCs are capable of migrating to lymph nodes and subsequent antigen presentation to T cells, initiating and shaping the adaptive immune response. Plasmacytoid DCs are not phagocytic and inefficient at antigen presentation. They are thought to play an important role in the immune response to viruses as they produce large amounts of type 1 interferon upon viral encounter. Langerhans cells are tissue-resident DCs in the skin that resemble tissue-resident macrophages in many ways but are also capable of migrating to lymphoid tissues (Satpathy *et al*. 2012, Pearce & Everts 2015).

Thyroid hormone metabolism in neutrophils

Intracellular thyroid hormone metabolism in neutrophils

Neutrophils contain essential elements required for intracellular TH metabolism and action. Murine neutrophils contain the TH transporter MCT8, whereas human neutrophils express MCT10 but not MCT8 mRNA (Boelen *et al*. 2005, van der Spek *et al*. 2016). It has long been known that activated neutrophils are capable of deiodinating both T_3 and T_4 (Woeber 1971, 1978, Woeber *et al*. 1972, Klebanoff & Green 1973, Woeber & Ingbar 1973). Research from the seventies already found that phagocytosing human neutrophils can generate both T_3 and rT_3 from T_4 and that this deiodinating activity was mainly present in the granule fraction of the cells (Woeber 1976, 1978). Neutrophils were also shown to contain saturable nuclear-binding sites for T_3 (Woeber 1977). It has since been found that type 3 deiodinase (D3), the TH-inactivating enzyme, is present in both human and murine neutrophils and is located in the cytosol and in bactericidal granules within the cell (Boelen *et al*. 2005, 2008, van der Spek *et al*. 2016). Human neutrophils were also recently shown to express type 1 deiodinase (D1) and TRα1 at the transcriptional level (van der Spek *et al*. 2016).

Effects of thyroid hormones on neutrophil function

Neutrophil bacterial killing can be mediated by a number of different mechanisms one of which is the generation of reactive oxygen species (ROS) by the NADPH oxidase system (Kolaczkowska & Kubes 2013). Circulating TH levels affect ROS generation by stimulated neutrophils. Hyperthyroidism results in increased ROS generation by stimulated neutrophils *ex vivo* compared to cells from euthyroid controls, whereas hypothyroidism has the opposite effect and limits neutrophil ROS generation. This has been demonstrated in neutrophils from hypothyroid and hyperthyroid rats (Videla *et al*. 1993, Fernandez & Videla 1995) and hyperthyroid and hypothyroid patients (Videla *et al*. 1993, Szabo *et al*. 1996, Russo-Carbolante *et al*. 2005*b*, Marino *et al*. 2006) or healthy controls with experimentally induced hyperthyroidism (Magsino *et al*. 2000). In both hypothyroidism and

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hyperthyroidism, the changes in neutrophil ROS generation are (partially) reversed by restoring TH levels to within the normal range (Videla *et al.* 1993, Marino *et al.* 2006).

Although studies using cells derived from hyperthyroid or hypothyroid patients and animals all find similar effects of TH levels on ROS generation, the effects of *in vitro* incubation of neutrophils with TH are less consistent. Some authors find that *in vitro* TH stimulation increases neutrophil ROS generation (Mezosi *et al.* 2005), whereas others only find an effect for supraphysiological levels of T_4 (Marino *et al.* 2006). In contrast, a decrease in ROS generation after TH incubation (Aoyagi *et al.* 1991) or no effect at all has also been reported (Videla *et al.* 1993). These conflicting results suggest that the effects of TH on neutrophil ROS generation cannot be entirely explained by direct effects of TH on these cells. The link between TH metabolism and oxidative stress that occurs outside innate immune cells has been reviewed elsewhere and is beyond the scope of this review (Mancini *et al.* 2016).

Another important neutrophil-killing mechanism is the use of antibacterial proteins housed in granules within the cell (Kolaczkowska & Kubes 2013). One of these proteins is myeloperoxidase (MPO). The only two studies to assess the effect of TH levels on MPO both find an increase in MPO activity in neutrophils either derived from hyperthyroid animals (Fernandez & Videla 1995) or incubated with TH *in vitro* (Mezosi *et al.* 2005).

Circulating TH levels appear to have a clear effect on neutrophil function. Mezosi and coworkers found that these effects were mediated via non-genomic pathways. The increase in neutrophil ROS production elicited by TH incubation *in vitro* was partially mediated via an unknown G-protein-coupled receptor and dependent on signalling through the protein kinase C pathway and increased intracellular Ca^{2+} levels (Fig. 1) (Mezosi *et al.* 2005). Hyperthyroidism did not affect superoxide dismutase activity and glutathione content indicating that the increase in ROS generation found was not due to changes in antioxidant defences (Russo-Carbolante *et al.* 2005*b*).

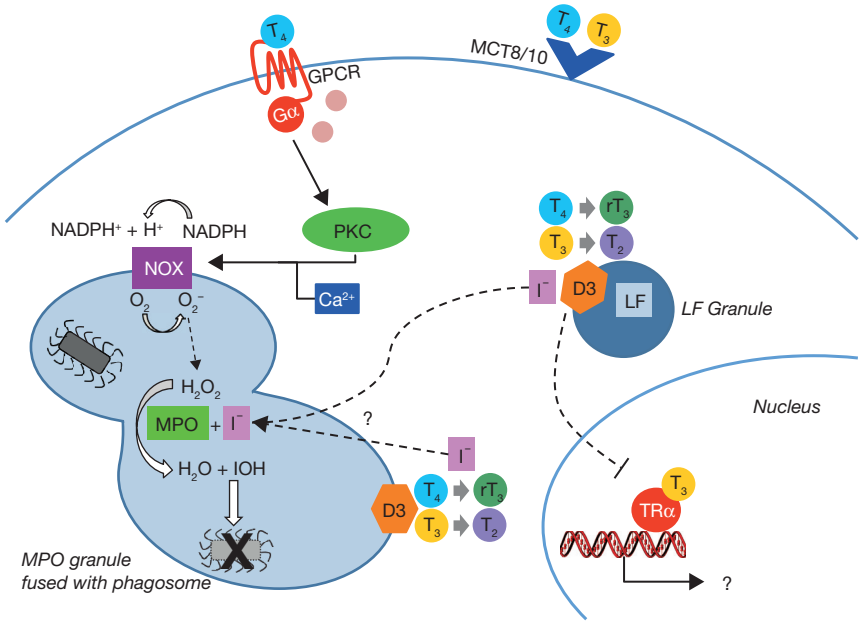

Figure 1
Hypothetical pathways explaining the effects of thyroid hormone on neutrophil NAPDH oxidase activity and bacterial killing. Thyroid hormone induces neutrophil NADPH oxidase (NOX) activity, resulting in increased production of reactive oxygen species. This phenomenon is thought to be mediated via a non-genomic pathway involving binding of TH to a G-protein-coupled receptor (GPCR), which induces NAPDH oxidase activity. This effect is dependent on protein kinase C (PKC) and adequate intracellular Ca^{2+} levels (Mezosi *et al.* 2005). Intracellular thyroid hormone metabolism may also play a role in neutrophils during bacterial killing. The thyroid hormone-inactivating type 3 deiodinase (D3) is present in murine and human neutrophils (Boelen *et al.* 2005, 2008, van der Spek *et al.* 2016). Mice that lack this enzyme suffer from impaired bacterial killing (Boelen *et al.* 2009). D3 is located in the cytoplasm and in granules containing either myeloperoxidase (MPO) or lactoferrin (LF) (van der Spek *et al.* 2016). TH enters the neutrophil via transporters (MCT8 or MCT10) where it is inactivated by D3, which removes an iodine atom from the inner ring of the hormone, converting T_4 to reverse (r)T_3 and T_3 to T_2. Increased D3 activity therefore results in decreased intracellular levels of T_3 together with the production of free iodide (I^-). One hypothesis explaining the role of D3 in microbial killing is that the iodide produced by D3 is utilized by MPO together with hydrogen peroxidase (H_2O_2) to generate hypoiodite (IOH), a toxic compound that is capable of killing bacteria (Klebanoff 1967, Boelen *et al.* 2011). The reduction of intracellular T_3 levels could theoretically also result in altered gene transcription, but no TH-responsive genes have been found in neutrophils yet.

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Role of intracellular thyroid hormone metabolism in neutrophil function

Besides the effects of circulating TH levels on neutrophils, intracellular TH metabolism appears to play an essential role in neutrophil function during infection and inflammation.

THs are drawn to the site of bacterial infection (Adelberg *et al.* 1971). Activated phagocytosing neutrophils are capable of cleavage of thyroxine-binding globulin (TBG), thus increasing the amount of extracellularly available T_4 (Jirasakuldech *et al.* 2000). As mentioned previously, phagocytosing neutrophils also metabolize significant amounts of TH (Woeber 1971, 1978, Woeber *et al.* 1972, Klebanoff & Green 1973, Woeber & Ingbar 1973). This suggests that TH metabolism plays an important role in infiltrating neutrophils during infection.

Most authors find that metabolism of TH by phagocytosing neutrophils results in the production of inorganic iodide, suggesting the involvement of the deiodinase enzymes (Woeber *et al.* 1972, Klebanoff & Green 1973, Woeber & Ingbar 1973). Other authors have found that phagocytosing neutrophils are capable of ether-linked cleavage of T_4, which results in the formation of diiodotyrosine (DIT) (Burger *et al.* 1983). The degradation of TH requires the intracellular formation of ROS as neutrophils from patients with chronic granulomatous disease, which is characterized by defective NAPDH oxidase resulting in reduced ROS generation, have significantly impaired ability to degrade TH (Klebanoff & Green 1973, Woeber & Ingbar 1973, Burger *et al.* 1983). Although isolated MPO is capable of degrading TH *in vitro*, neutrophils from MPO-deficient patients degrade TH to the same degree as controls suggesting that the degradation of TH by leukocytes is not MPO dependent *in vivo* (Klebanoff & Green 1973, Woeber & Ingbar 1973, Burger *et al.* 1983).

Type 3 deiodinase (D3) is expressed in infiltrating murine neutrophils during both bacterial infection and sterile inflammation (Boelen *et al.* 2005, 2008). It was recently shown to also be present in human neutrophils (van der Spek *et al.* 2016). In a murine model for chronic local inflammation in which mice were injected with turpentine resulting in the formation of a subcutaneous abscess, D3 activity was strongly elevated in inflamed tissue compared to control tissue (Boelen *et al.* 2005). Mice that lack D3 have impaired bacterial killing upon infection with *Streptococcus pneumoniae* (Boelen *et al.* 2009). D3 in human neutrophils was found in intracellular granules involved in bacterial killing (van der Spek *et al.* 2016). The enzyme was also found in early-stage neutrophil extracellular traps (NETs) (van der Spek *et al.* 2016). Together these data suggest that D3 is important for neutrophil function during infection and inflammation. The mechanism behind this is currently unknown. We have previously suggested as a possible explanation that the iodide produced by D3 could be used by the MPO system together with H_2O_2 to generate hypoiodite, a toxic compound that is capable of killing bacteria (Fig. 1) (Klebanoff 1967, Boelen *et al.* 2011).

Type 1 deiodinase is also present in human neutrophils, whereas its expression in murine neutrophils is unknown (van der Spek *et al.* 2016). D1 could also potentially be a source of iodide for the cells; however, blocking D1 activity by PTU was shown to have no effect on the neutrophil respiratory burst, which is in contrast to the observation that TH raises neutrophil ROS production (Mezosi *et al.* 2005, Russo-Carbolante *et al.* 2005*a*).

Thyroid hormone metabolism in monocytes and macrophages

Intracellular thyroid hormone metabolism in macrophages

Macrophages contain several essential elements of intracellular TH metabolism. Both macrophage cell lines and human and murine microglia contain TH transporters. Macrophage cell lines predominantly express MCT10 and to a lesser extent MCT8 (Kwakkel *et al.* 2014). Microglia, the resident macrophages of the brain, contain the TH transporters LAT2, MCT10 and OATP4a1 (Wirth *et al.* 2009, Braun *et al.* 2011). Macrophages were also found to express D2 (Kwakkel *et al.* 2014), TRα1 and possibly also TRβ although authors have reported conflicting results (Billon *et al.* 2014, Kwakkel *et al.* 2014, Lourbopoulos *et al.* 2014, Perrotta *et al.* 2014). Several recent papers have demonstrated that both human and murine macrophages are able to produce a functional TSHβ splice variant that is positively regulated by T_3 and capable of stimulating the TSH receptor (Vincent *et al.* 2009, Baliram *et al.* 2013, 2016). It has been suggested that this TSHβ splice variant plays a role in bone physiology; however, whether it affects macrophage function is currently unknown.

Effects of extracellular thyroid hormone levels on macrophage function

There are several studies available on the effects of TH administration on macrophage function either *in vivo*,

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ex vivo or *in vitro*. The majority of these functional studies assessed pro-inflammatory macrophage function.

Several studies in both human subjects and animal models have found that, similar to neutrophils, stimulated hyperthyroid macrophages have increased ROS production *ex vivo* (Videla *et al*. 1993, Magsino *et al*. 2000) although one study found an opposite effect (Rosa *et al*. 1995). PTU treatment of hyperthyroid patients normalized ROS production although these findings could not be replicated *in vitro* (Videla *et al*. 1993). It should be noted that the majority of these studies assessed a mixed population of mononuclear cells containing significant numbers of other cell types, making it impossible to determine the contribution of the macrophage/monocyte subset. Recently, TH administration was found to increase iNOS expression, nitrite production and *in vitro* bacterial killing in both a human and a mouse macrophage cell line and treatment with TH increased the survival after meningococcal infection in mice (Chen *et al*. 2012).

Macrophage phagocytosis is also affected by TH concentrations with most studies finding that higher levels of available TH result in an increased phagocytic capacity. Strenuous exercise leads to both increased plasma TH levels and increased macrophage phagocytosis (Forner *et al*. 1996, Ortega *et al*. 1996). This effect was confirmed *in vitro* where incubation of

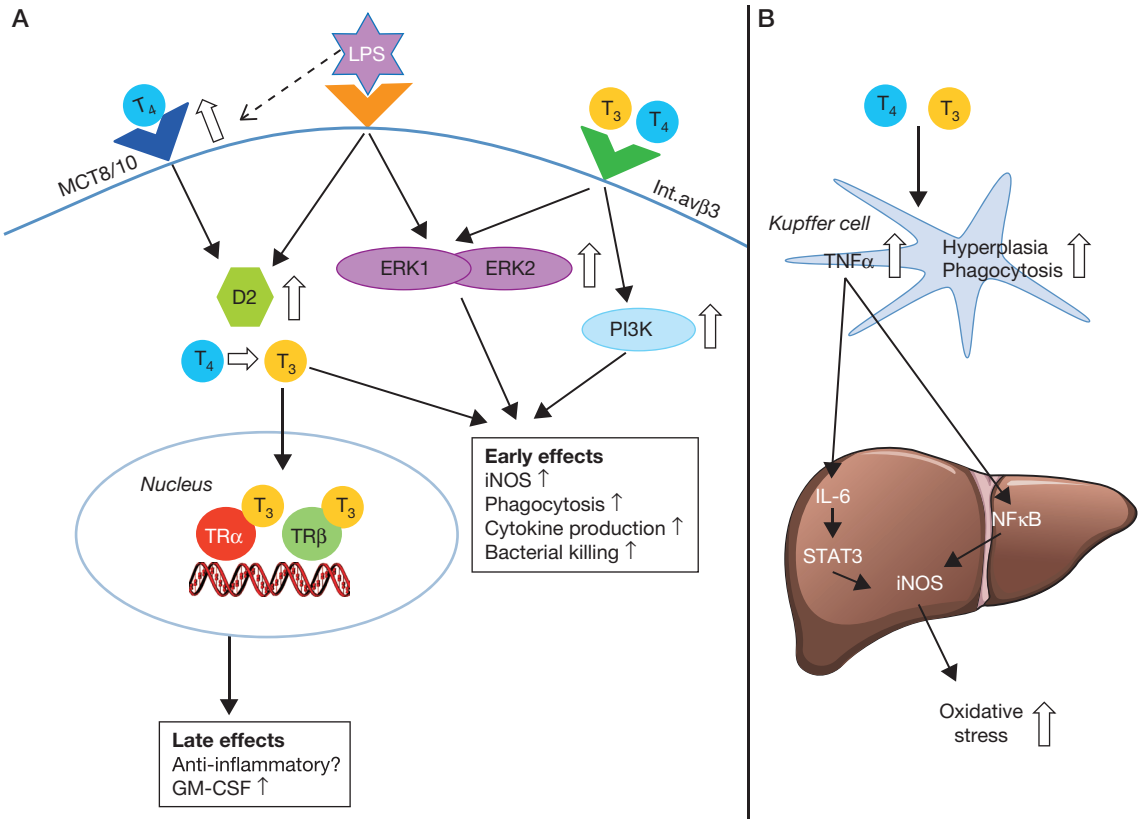

Figure 2
Thyroid hormone induces a pro-inflammatory response in macrophages. (A) The effects of TH in macrophages are mediated through integrin αvβ3 or through modulation of intracellular TH levels. TH can bind to integrin αvβ3 on the macrophage cell surface resulting in the activation of PI3K and ERK1/2 pathways followed by the upregulation of inducible nitric oxide synthase (iNOS) (Chen *et al*. 2012). Alternatively, TH can enter the cell through TH transporters MCT8 or MCT10 after which the prohormone T_4 is converted to active hormone T_3 by type 2 deiodinase (D2) resulting in increased phagocytosis and cytokine response. D2 is induced in lipopolysaccharide (LPS)-stimulated macrophages (Kwakkel *et al*. 2014). The effects of intracellular T_3 are partly mediated via TRα, which is required for adequate macrophage function (Billon *et al*. 2014, Kwakkel *et al*. 2014). Low-grade inflammation found in unstimulated TRα-knockout macrophages suggests that TRα possibly attenuates the rapid pro-inflammatory response generated by increased intracellular TH levels (Billon *et al*. 2014). (B) Kupffer cells are the resident macrophages of the liver. TH stimulation *in vivo* results in Kupffer cell hyperplasia and enhanced phagocytosis (Tapia *et al*. 1997, Valencia *et al*. 2004). TH transporter and receptor expression in Kupffer cells have not yet been studied. TH also increases the production of TNFα by Kupffer cells (Valencia *et al*. 2004, Fernandez *et al*. 2005, 2007*b*). TNFα produced by Kupffer cells results in liver NFκB activation (Valencia *et al*. 2004). IL-6 production is also increased, resulting in increased STAT3 activation (Tapia *et al*. 2006). The activation of both these pathways results in increased iNOS activity in the liver, resulting in the production of larger amounts of reactive oxygen species and hepatic oxidative stress (Fernandez *et al*. 2005).

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murine peritoneal macrophages with TH also resulted in increased phagocytosis and chemotaxis (Forner *et al.* 1996, Ortega *et al.* 1996, 1999). Notably, a 10,000-fold greater concentration of TH did not affect macrophage function, suggesting that this effect is only present at physiological levels (Forner *et al.* 1996, Ortega *et al.* 1999). One study found an opposite effect on phagocytosis with macrophages from hypothyroid animals demonstrating higher phagocytosis and no effect of hyperthyroidism on phagocytic capacity (Rosa *et al.* 1995).

The generally observed pro-inflammatory effect of TH administration on macrophages suggests a shift towards an M1 phenotype. Incubation with T_3 indeed polarizes bone marrow-derived murine macrophages towards a pro-inflammatory M1 phenotype and inhibits M2 polarization (Perrotta *et al.* 2014). Polarization was accompanied by a change in $TR\alpha_1$:$TR\beta_1$ ratio, suggesting that perhaps relative abundance of TR isoforms is associated with macrophage phenotype (Perrotta *et al.* 2014). It should be noted that these effects were achieved by incubating cells with supraphysiological levels of T_3 (500 nM); therefore, these results are in contrast with the studies mentioned previously that find no effect of supraphysiological TH levels (Forner *et al.* 1996, Ortega *et al.* 1999). This could be explained by the degree of excess: 500 nM is an approximately 250-fold higher concentration than circulating T_3 in euthyroid mice, whereas Forner and coworkers and Ortega and coworkers used a 10,000-fold higher concentration.

Role of intracellular thyroid hormone metabolism in macrophage function

Although the effects of extracellular thyroid hormone concentrations on macrophages are reasonably well characterized, the role of specific elements of intracellular TH metabolism in these effects has only recently been studied. The results indicate that adequate regulation of intracellular TH levels affects macrophage function via a combination of genomic and non-genomic pathways. The TH-induced increase in iNOS expression and activity, phagocytosis and bacterial killing is thought to be partly mediated via binding of TH to integrin αvβ3 on the extracellular surface of the cell, which results in the rapid activation of the PI3K and ERK1/2 signalling pathways (Fig. 2A) (Chen *et al.* 2012).

Besides the extracellular binding of TH, regulation of intracellular TH levels was also recently shown to play an essential role in the pro-inflammatory response of macrophages (Kwakkel *et al.* 2014). D2, which converts T_4 to T_3 thereby regulating intracellular TH bioavailability, is induced in macrophages stimulated with bacterial endotoxin (lipopolysaccharide; LPS) together with TRα1 and MCT10, indicating a shift towards increased TH action during inflammation (Fig. 2A) (Kwakkel *et al.* 2014). Furthermore, D2 knockdown resulted in impaired macrophage phagocytosis and blunted cytokine response to LPS stimulation (Kwakkel *et al.* 2014). These effects appear to be partly mediated via genomic pathways as knockout of TRα, which is the predominant TR isoform in macrophages, also results in aberrant macrophage function (Billon *et al.* 2014, Kwakkel *et al.* 2014). Macrophages from TRα-knockout mice have impaired cholesterol efflux during atherosclerosis resulting in earlier plaque formation (Billon *et al.* 2014). Furthermore, macrophages that lack TRα demonstrate low-grade inflammation at baseline compared to controls, indicating an anti-inflammatory role for TRα (Billon *et al.* 2014). This suggests that attenuation of the rapid pro-inflammatory response generated by increased intracellular TH levels could be mediated via TRα (Fig. 2A).

Thyroid hormone metabolism in tissue-resident macrophages

Tissue-resident macrophages can vary widely in phenotype depending on which tissue they are from. TH metabolism has been specifically investigated in two well-characterized types of tissue-resident macrophages: Kupffer cells and microglia.

Kupffer cells As the resident macrophages of the liver, Kupffer cells are essential for liver homeostasis. This role is enhanced during infection and inflammation. Several studies by the same group have analysed the effect of TH administration on rat liver and the role of Kupffer cells in this process. T_3 administration induces oxidative stress in the liver (Tapia *et al.* 1997, 2006, 2010, Valencia *et al.* 2004, Fernandez *et al.* 2005, 2007*a*,*b*, 2008, 2009). This is thought to be mediated via Kupffer cells, which demonstrate hyperplasia, increased phagocytic capacity, increased ROS generation and tumour necrosis factor α (TNFα) production in response to T_3 administration *in vivo* (Fig. 2B) (Tapia *et al.* 1997, Valencia *et al.* 2004, Fernandez *et al.* 2005, 2007*b*). Kupffer cell activation by T_3 triggers a cascade of responses in the liver including increased TNFα and interleukin-6 (IL-6) levels, which lead to the activation of liver STAT3 phosphorylation and nuclear factor kappa-B (NFκB) DNA binding. This ultimately increases liver iNOS expression and activity

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together with other markers of oxidative stress including decreased liver glutathione content (an important antioxidant) and higher protein oxidation (Fig. 2B) (Tapia *et al*. 1997, 2006, Valencia *et al*. 2004, Fernandez *et al*. 2005, 2007*a*,*b*). Conversely, another study found that T_3 inhibited STAT3 signalling in macrophages after LPS or IL-6 stimulation and had no effect on TNFα induction and NFκB activation *in vitro* (Contreras-Jurado *et al*. 2016). Both acute and chronic inflammation results in increased liver D2 mRNA expression and activity (Kwakkel *et al*. 2014). This increase occurs independently of changes in serum TH levels and is thought to be caused by increased Kupffer cell activation resulting in higher D2 activity. This suggests that Kupffer cells are also capable of local T_3 generation during inflammation, which could be an important mediator in the inflammatory response of these cells (Kwakkel *et al*. 2014).

The exact pathways through which the effects of T_3 are mediated in Kupffer cells are still unclear. It is possible that immediate non-genomic actions of TH are partly attenuated by late genomic TR effects that inhibit the initial pro-inflammatory response to TH in these cells.

Interestingly, the Kupffer cell-mediated induction of hepatic oxidative stress by T_3 appears to ameliorate the harmful effects of ischaemic reperfusion injury in the liver. Hepatic ischaemic reperfusion injury in rats results in severe liver damage (Fernandez *et al*. 2007*a*, 2008, 2009, Tapia *et al*. 2010). These effects were prevented and even reversed by preconditioning with a single dose of T_3 (Fernandez *et al*. 2007*a*, 2008, 2009, Tapia *et al*. 2010). The effects of T_3 preconditioning were abolished by the addition of treatment with N-acetylcysteine, a powerful antioxidant, suggesting that the protective effect of T_3 on liver ischaemic reperfusion injury is achieved through

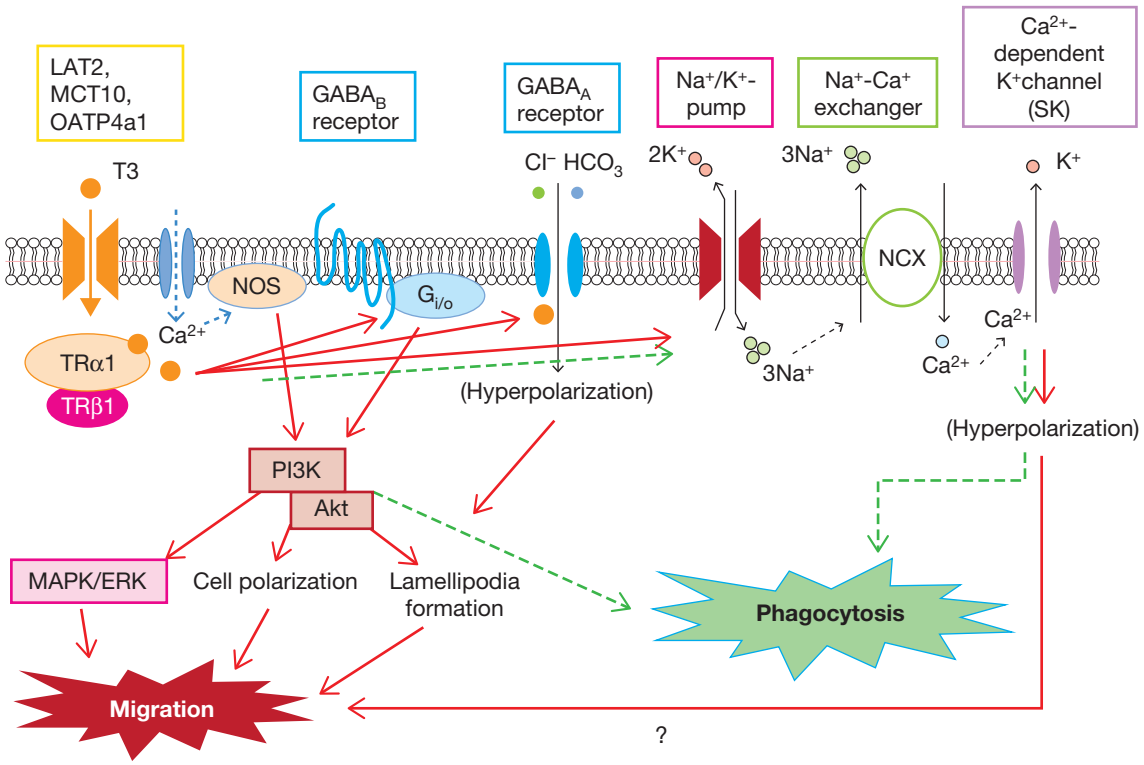

Figure 3
Effects of thyroid hormone on microglia (reproduced with permission from Mori *et al*. 2015). Schematic representation of possible signal transduction pathways activated by T_3. Microglia contain various TH transporters (LAT2, OATP4a1 and MCT10) and TH receptors. Intracellular T_3 activates intracellular TRs (TRα1 and possibly TRβ1) and appears to also couple to various other factors, such as nitric oxide synthase (NOS), $G_{i/o}$-protein, PI3K and MAP/ERK. The receptors for gamma-aminobutyric acid (GABA) A and B are also involved in T_3-induced microglial migration but not in T_3-induced phagocytosis, the mechanism of which is unclear. The reverse mode of the Na^+/Ca^{2+} exchanger (NCX) may be activated by Na^+ influx by the Na^+/K^+ pump, resulting in Ca^{2+} influx, which in turn activates small-conductance Ca^{2+}-dependent K^+ channels (SK). This subsequently induces microglial migration and phagocytosis, possibly due to membrane hyperpolarization. Hypothetical signalling pathways for phagocytosis are indicated by the dotted line. Reproduced, with permission, from Mori Y, Tomonaga D, Kalashnikova A, Furuya F, Akimoto N, Ifuku M, Okuno Y, Beppu K, Fujita K, Katafuchi T, *et al*. (2015) Effects of 3,3′,5-triiodothyronine on microglial functions, *Glia*, volume **63**, pages 906–920. Copyright (2015) Wiley Periodicals, Inc.

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development of transient and reversible oxidative stress (Fernandez *et al*. 2008, 2009).

Microglia Microglia are the resident macrophages of the central nervous system and are derived from myeloid progenitor cells that migrate to the brain during foetal development (Mass *et al*. 2016). Due to the neurological phenotype associated with mutations in TH transporters, intracellular TH metabolism in brain cells has been assessed in detail. Both murine and human microglia express high levels of LAT2 in addition to MCT10 and OATP4a1, allowing them to transport TH into the cell (Wirth *et al*. 2009, Braun *et al*. 2011). In addition, microglia express both TRα_1 and TRβ_1 (Lima *et al*. 2001).

Physiological TH concentrations appear to be crucial for microglial growth and morphological differentiation (Lima *et al*. 2001). Hypothyroid rats showed delayed microglial growth and differentiation, whereas hyperthyroid animals displayed the opposite phenotype with accelerated microglial growth and differentiation (Lima *et al*. 2001). A recent study by Mori and coworkers assessed in detail the effects of T_3 on microglial activation and the signalling pathways involved (Mori *et al*. 2015). T_3 exposure increased migration, activation and phagocytosis in primary mouse microglia *in vitro*, indicating a shift towards a more mature and pro-inflammatory phenotype (Mori *et al*. 2015). These effects were found to be mediated via both genomic and non-genomic pathways (detailed in Fig. 3; reproduced with permission from Mori *et al*. 2015). Microglial migration and morphological changes associated with cell activation were dependent not only on TH transporters and TRs but also on gamma-aminobutyric acid (GABA)-A and GABA-B receptors, NOS, intracellular Ca^{2+} influx and G-protein-mediated signalling pathways including phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) (Mori *et al*. 2015). Conversely, the T_3-induced stimulation of phagocytosis appeared to be partly mediated by other pathways that did not involve the GABA-A and GABA-B receptors (Mori *et al*. 2015).

Thyroid hormone metabolism in dendritic cells

Intracellular thyroid hormone metabolism in dendritic cells

DCs are known to express TRβ1 and to a lesser degree TRα1 (Mascanfroni *et al*. 2008). Furthermore, it was recently

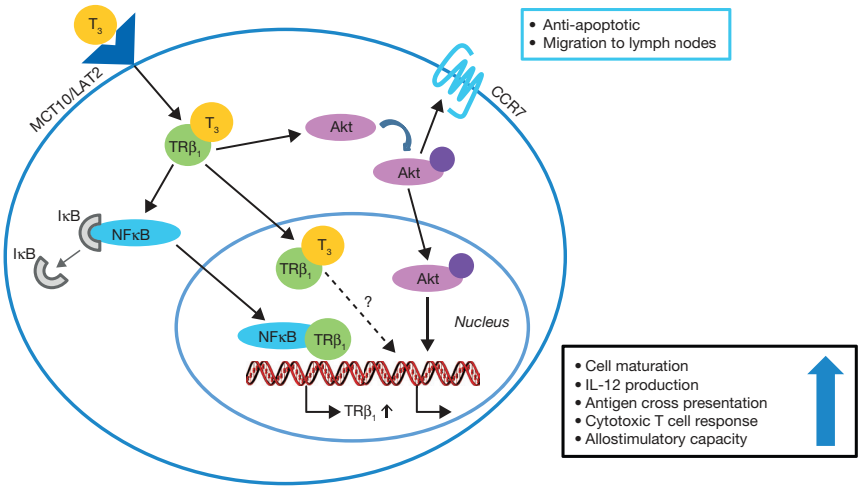

Figure 4
Thyroid hormone enhances dendritic cell maturation and function. In dendritic cells, T_3 enters the cell via TH transporters MCT10 or LAT2 and binds to cytoplasmic TRβ1 (Gigena *et al*. 2015 abstract 475, presented at the International Thyroid Congress). Upon binding of T_3, TRβ1 translocates from the cytoplasm to the nucleus (Mascanfroni *et al*. 2008, 2010). T_3 binding to TRβ1 also activates cytoplasmic NFκB by initiating degradation of IκB so that NFκB can translocate to the nucleus and regulate gene transcription, including induction of TRβ1 transcription, which is an NFκB target gene, thus forming a regulatory feedback loop controlling TRβ1 levels (Mascanfroni *et al*. 2008). Furthermore, binding of T_3 to cytoplasmic TRβ1 activates the Akt pathway, leading to a nuclear shift of phosphorylated Akt and increased chemokine (C–C motif) receptor type 7 (CCR7) expression, which prolongs cell viability and augments cell migration towards lymph nodes (Mascanfroni *et al*. 2010, Alamino *et al*. 2015). T_3 stimulation of DCs shifts the cells towards a more pro-inflammatory phenotype through induction of cell maturation, increased IL-12 production, improved antigen cross presentation and an enhanced ability to stimulate a cytotoxic T-cell response and trigger antigen-specific responses *in vivo* (Mascanfroni *et al*. 2008, 2010, Alamino *et al*. 2015, 2016).

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reported that DCs are capable of active TH transport via transporters MCT10 and LAT2 and exhibit D2 and D3 enzymatic activity (Gigena *et al*. 2015 abstract 475, presented at the International Thyroid Congress).

Effects of extracellular thyroid hormone levels on dendritic cell function

TH stimulation has profound effects on DC phenotype. Incubation of human peripheral blood mononuclear cells with TH enhances their ability to differentiate into functional DCs (Mooij *et al*. 1994). Extensive work by the Pellizas group has shown that stimulation of murine bone marrow-derived DCs with physiological levels of T_3 results in the initiation of the adaptive immune response by induction of DC maturation, increased interleukin-12 (IL-12) production, improved antigen cross presentation and enhanced DC ability to stimulate a cytotoxic T-cell response and trigger antigen-specific responses *in vivo* (Fig. 4) (Mascanfroni *et al*. 2008, 2010, Alamino *et al*. 2015). Cell survival and ability to migrate to lymph nodes *in vivo* are also enhanced (Alamino *et al*. 2015). The effects of T_3 stimulation are mediated via the TRβ1 receptor and the Akt and NFκB pathways independently of the PI3K pathway (Fig. 4) (Mascanfroni *et al*. 2008, 2010). The promoter region of the TRβ1 gene contains an NFκB response element, which upregulates TRβ1 expression after T_3 stimulation, suggesting a regulatory feedback loop (Mascanfroni *et al*. 2010). Dexamethasone potently inhibits and even reverses the effects of T_3 in DCs (Montesinos *et al*. 2012).

The effect of T_3 on DCs could be beneficial in anti-cancer vaccines. As DCs are antigen-presenting cells, a patient's own DC can be loaded with tumour antigen *ex vivo*, inducing DC maturation (Palucka & Banchereau 2013). The DCs are then re-administered to the patient resulting in a cytotoxic T-cell response against the tumour. Unfortunately, the effects of DC-based vaccines are frequently limited by the short lifespan of activated mature DCs and the risk of immune tolerance (Palucka & Banchereau 2013). This necessitates the use of costimulatory molecules that increase DC survival and immunogenicity. As T_3 increases DC survival and DC ability to migrate to lymph nodes, T_3 could potentially be of use in DC-based anti-cancer vaccines (Alamino *et al*. 2016). Indeed, vaccination with T_3-stimulated DCs in a mouse melanoma model inhibited tumour growth and increased host survival (Alamino *et al*. 2015).

Summary

Neutrophils, macrophages and dendritic cells are cells of the innate immune system that are crucial for the host defence against invading pathogens. Thyroid hormone plays an important role in the function of these cells. Neutrophils, macrophages and dendritic cells have all been shown to contain essential elements required for intracellular TH metabolism and TH action, including TH transporters, deiodinases and TRs. Furthermore, circulating TH levels strongly affect innate immune cell function. In general, incubation with TH appears to have a pro-inflammatory effect in these cells. This is illustrated by the fact that ROS production and MPO activity are increased in hyperthyroid neutrophils (Videla *et al*. 1993, Fernandez & Videla 1995, Szabo *et al*. 1996, Magsino *et al*. 2000, Mezosi *et al*. 2005, Russo-Carbolante *et al*. 2005*b*, Marino *et al*. 2006). Higher TH levels also increase reactive nitrogen species production, phagocytosis and bacterial killing in macrophages (Forner *et al*. 1996, Ortega *et al*. 1996, Chen *et al*. 2012). In accordance with these effects, T_3 has been shown to polarize macrophages towards a pro-inflammatory M1 phenotype, whilst inhibiting anti-inflammatory M2 markers (Perrotta *et al*. 2014). In dendritic cells, TH administration also has pro-inflammatory effects, illustrated by increased cell maturation, pro-inflammatory cytokine production and the increased ability to elicit a cytotoxic T-cell response (Alamino *et al*. 2015).

Although the effects of circulating thyroid hormone levels on innate immune cells have been studied for decades, the mechanisms involved have only recently been assessed and are currently only partially understood. In all three cell types discussed, there appears to be an interplay of genomic and non-genomic pathways involved in the effects of TH on cellular function. In neutrophils, extracellular TH appears to induce its pro-inflammatory effects by binding to an unknown G-protein-coupled receptor whose downstream effects are mediated via the protein kinase C pathway (Fig. 1) (Mezosi *et al*. 2005). In macrophages, another non-genomic pathway has been described involving binding of extracellular TH to integrin αvβ3, resulting in activation of the ERK and PI3K signalling pathways (Fig. 2) (Chen *et al*. 2012). The effects of T_3 stimulation in dendritic cells are mediated via $TR\beta_1$ and the Akt and NFκB pathways (Fig. 4) (Mascanfroni *et al*. 2010). Besides the clear effects of altered extracellular TH levels, recent research has shown that several elements of intracellular TH metabolism

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appear to be essential for adequate pro-inflammatory neutrophil and macrophage function. In neutrophils, the thyroid hormone-inactivating enzyme D3 appears to play an important role during infection (Boelen *et al*. 2005, 2009, van der Spek *et al*. 2016). D3 induction results in decreased intracellular TH bioavailability and increased rT_3 levels, so the role of D3 during bacterial killing may appear contradictory given the effects of exogenous TH in these cells. However, D3 is a T_3-responsive gene that is known to be induced by T_3 in other tissues (Hernandez 2005). Whether a similar mechanism is present in neutrophils has not been studied to date, but this could potentially explain the similarity between the effects of higher extracellular TH levels and intracellular D3 activity. In macrophages, both D2 and $TR\alpha_1$ are required for correct cellular function, which is accordance with the effects of extracellular TH as both these mechanisms result in increased intracellular TH action (Billon *et al*. 2014, Kwakkel *et al*. 2014). These studies convincingly demonstrate that adequate regulation of intracellular TH bioavailability is essential for neutrophil and macrophage function.

Conclusions

Circulating TH levels have a profound effect on neutrophil, macrophage and dendritic cell function. In general, increased TH levels result in an amplification of the pro-inflammatory response of these cells. Besides a pro-inflammatory effect of extracellular TH, the cellular response to pro-inflammatory stimuli appears to be dependent on functional intracellular TH metabolism, suggesting that TH metabolism plays an important role in host defence against infection. To date, this has best been demonstrated in macrophages.

Future research should focus on the intracellular pathways involved in the modulation of the immune response of innate immune cells by TH. Although a small number of recent promising studies have analysed the pathways that mediate the effects of TH stimulation in innate immune cells and the role of intracellular TH metabolism in innate immune cell function, still relatively little is known about the mechanisms involved. Whether the effects of increased TH levels and the functional role of intracellular TH metabolism in the immune response of these cells are in fact linked and part of the same mechanisms remains to be studied. Studies focussing on these issues would further elucidate the important connection between the endocrine and innate immune systems. This overview of the literature suggests that TH plays an important role in the host defence against infection through the modulation of innate immune cell function.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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