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Different subsets of natural killer T cells may vary in their roles in health and disease

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Summary

Natural killer T cells (NKT) can regulate innate and adaptive immune responses. Type I and type II NKT cell subsets recognize different lipid antigens presented by CD1d, an MHC class-I-like molecule. Most type I NKT cells express a semi-invariant T-cell receptor (TCR), but a major subset of type II NKT cells reactive to a self antigen sulphatide use an oligoclonal TCR. Whereas TCR-α dominates CD1d-lipid recognition by type I NKT cells, TCR-α and TCR-β contribute equally to CD1d-lipid recognition by type II NKT cells. These variable modes of NKT cell recognition of lipid–CD1d complexes activate a host of cytokine-dependent responses that can either exacerbate or protect from disease. Recent studies of chronic inflammatory and autoimmune diseases have led to a hypothesis that: (i) although type I NKT cells can promote pathogenic and regulatory responses, they are more frequently pathogenic, and (ii) type II NKT cells are predominantly inhibitory and protective from such responses and diseases. This review focuses on a further test of this hypothesis by the use of recently developed techniques, intravital imaging and mass cytometry, to analyse the molecular and cellular dynamics of type I and type II NKT cell antigen-presenting cell motility, interaction, activation and immunoregulation that promote immune responses leading to health versus disease outcomes.

Keywords: antigen-presenting cells; CD1; cytokines; dendritic cells; glycolipids; innate immunity; myeloid-derived suppressor cells; natural killer T cells; sulphatide; T-cell receptor.

Introduction

Pivotal to the outcome of immune responses in health and disease are the function and activity of different immune cell types that mediate immunosuppression and immunoregulation. These cell types include regulatory T (Treg) cells, myeloid-derived suppressor cells and natural killer T (NKT) cells. In this review, we focus primarily on analyses of the activity and function of NKT cells, which are innate-like and are comprised of two main subsets, type I and type II NKT cells.[1–4] Both subsets of NKT cells can play an important modulatory role in the induction and/or prevention of autoimmune disease, inflammation and cancer. From several recent reviews of the many immune responses mediated by type I and type II NKT cells in health and disease,[2–14] it is evident that our knowledge of NKT cell activity and function has advanced quite rapidly and significantly. Notwithstanding, we still have only a limited knowledge of where and how NKT cell–antigen-presenting cell (APC) interactions occur *in vivo*, and how they regulate a host of immune responses.

During an autoimmune disease, different subsets of NKT cells may increase or decrease in number and/or function at a particular anatomical site.[8] Results obtained from studies of experimental animal models of autoimmune disease and inflammation provide the basis of a hypothesis that addresses three main properties of NKT cells during such responses (Table 1). First, type I NKT cells can be either pathogenic or protective. Second, type I NKT cells have a greater propensity to be more pathogenic than protective. Third, type II NKT cells function predominantly to protect from inflammation and autoimmune disease. A test of this hypothesis requires that the

Table 1. Hypothesis to explain the different functional roles of natural killer T (NKT) cell subsets in health and disease

1	During the development and progression of an inflammatory response and autoimmune disease, type I NKT cells have the capacity to function as both pathogenic and protective T cells
2	Depending on the molecular and cellular environment of NKT cells in a target tissue and the stage of disease development, NKT cells can adopt a preferential functional role. This may enable type I NKT cells to have a greater propensity to be more pathogenic than protective in a given disease or at a specific stage of disease development
3	The molecular and cellular environment and/or stage of disease development in a target tissue enables type II NKT cells to function predominantly to protect from autoimmune and inflammatory diseases

factors and mechanisms that give rise to these outcomes *in vivo* are determined. It is anticipated that the identification of the molecular and cellular factors that drive these mechanisms will facilitate the development of novel immunotherapeutic protocols to prevent and treat inflammation and autoimmune disease.

Hence, the objectives of this review are: (i) to provide novel insight into how type I and type II NKT cells may cross-talk with other immune cells to regulate immune responses, and (ii) to determine how such analyses may enhance the success of future clinical trials of type I and type II NKT cell antagonists in inflammation and autoimmune disease. First, we highlight recent clinical and experimental advances in our understanding of the lipid antigens, inflammatory milieu, innate-like mechanisms and cellular interactions that regulate the activation and interactions of NKT cell subsets. Next, we discuss the rationale for why the application of several novel techniques to analyses of NKT cell movement and function *in vivo* may provide more insight into the design of improved clinical trials of autoimmune disease.

Type I and type II NKT cell subsets

The NKT cells express T-cell antigen receptors (TCR) characteristic of conventional T cells and several cell surface proteins characteristic of NK cells, such as CD56/161 (humans) and NK1.1 (mice).[2,3,5] NKT cells are generally reactive to lipid antigens presented by CD1d MHC class I like molecules.[2–15] Depending on the target tissue, different types of APCs including dendritic cells (DCs), macrophages (Mϕ), B cells, thymocytes, adipocytes and hepatocytes, can express CD1d molecules and activate NKT cells. In this review, we focus on analyses of CD1d-mediated responses of the type I and type II NKT cell subsets. Notwithstanding, it should be kept in mind that additional MHC class I like molecules such as CD1a, CD1b, CD1c and CD1e, as well as MR1, are expressed on APCs and can activate various subsets of T cells. The latter types of CD1-restricted T-cell subsets are not discussed here.

The developmental mechanisms involved in the commitment and maturation of NKT cells employ transcription factors and genes distinct from and shared by both MHC-restricted T cells and NK cell lineages.[12] In a manner similar to that of the T helper type 1 (Th1), Th2, Th17, Treg and follicular helper subsets of MHC-restricted $CD4^+$ T cells, subsets of type I (iNKT) cells, have been identified.[11] Many transcription factors [e.g. promyelocytic leukaemia zinc finger, T box transcription factor (T-bet), retinoic acid receptor-related orphan receptor-γt and GATA-binding protein 3] that mediate the development of MHC-restricted $CD4^+$ T-cell subsets also function in type I NKT cell subsets. The acquisition of expression of NK receptors by NKT cells during thymic maturation is driven by the transcription factor T-bet.[13] However, it is not yet known whether plasticity (change in function in response to an experience) is manifested among the type I NKT cell subsets. This section will focus primarily on the functional roles of the type I and type II NKT cell subsets.

Activation of type I NKT cells with a strong agonist such as α-galactosylceramide (αGalCer), an exogenous marine-derived glycolipid, stimulates the rapid release of many cytokines that elicit both Th1 [interferon-γ (IFN-γ)] and Th2 [interleukin-4 (IL-4) and IL-13] responses.[6–17] The widely studied type I NKT cells are more prevalent than type II NKT cells in mice than in humans,[1,18,19] and comprise about 50% of murine intrahepatic lymphocytes.[20–22] A major difference between the two subsets resides in their TCRs. The type I NKT cell invariant TCR is encoded predominantly by a germline Vα gene (75–88%) (Vα14/Jα18 in mice and Vα24/JαQ in humans), as well as more diverse non-germline Vβ chain genes (Vβ8.2/7/2 in mice and Vβ11 in humans).[1–19,23–25] Type I NKT cells respond to both α- and β-linked glycolipids. The semi-invariant TCR on type I NKT cells binds to CD1d in a parallel configuration that mainly involves the α-chain.[2,4,15,24]

Whereas type II NKT cells comprise a minor subset in the mouse, they belong to a more predominant subset in humans.[1,26] A major proportion of type II NKT cells recognizes a naturally occurring self antigen known as sulphatide, which is enriched in several membranes, including myelin in the central nervous system (CNS), pancreas, kidney and liver (Table 2). Generally, sulphatide-reactive type II NKT cells mediate protection from autoimmune diseases by down-regulation of inflammatory responses elicited by type I NKT cells.[27,28] However, non-sulphatide-reactive type II NKT cells may play a pathogenic role in other diseases, such as ulcerative colitis.[29] Sulphatide-reactive type II NKT cells express oligoclonal TCRs by utilization of a limited number of Vα- and Vβ-chains. In contrast to type I NKT cells, only about

Table 2. Most common self-lipids recognized by natural killer T (NKT) cell subsets

	Type I NKT cells		Type II NKT cells	
Self-lipids	Mouse	Human	Mouse	Human
Sulphatide	−	−	+	+
iGB3	+	−	−	−
GD3	+	NT	NT	NT
βGlcCer	+	+	+	NT
βGalCer	−	−	+	NT
Lysophosphatidylethanolamine	−	NT	+	NT
Lysophosphatidylcholine	−	±	+	+
Lysosphingomyelin	−	+	−	−
Plasmalogen Lysophosphatidylethanolamine and Lysophosphatidic acid	+	+	NT	NT

+, reactivity of the majority of NKT cells; ±, reactivity of a low percentage (< 10%) of NKT cells; −, absence of reactivity of NKT cells; NT, not tested.

14% of TCR Vα and 13–27% of TCR Vβ chains in type II NKT cells are encoded by germline gene segments.[28] Notably, type II NKT TCRs contact their ligands primarily via their β-chain rather than the α-chain, suggesting that the TCR Vβ-chain contributes significantly to antigen fine specificity.[30] The mechanism of binding of type II NKT TCRs to antigens uses features of TCR binding shared by both type I NKT cells and conventional T cells.[1,28,30,31] Importantly, type I NKT cells but not type II NKT cells respond to α-linked glycolipids.[30] Hence, type I and type II NKT cell subsets display distinct modes of recognition and activation by CD1d-bound glycolipid antigens.

In addition to TCR-$\alpha\beta^+$ T cells, sulphatide-specific T cell lines derived from peripheral blood mononuclear cells (PBMCs) of both healthy subjects and patients with demyelinating diseases, e.g. multiple sclerosis (MS), express the Vδ1 variable gene segment that is rare in the blood and more abundant in MS lesions and the intestine.[32] Vδ1 TCRs from different individuals bind to CD1d–sulphatide complexes in a sulphatide-specific manner. These findings suggest that human Vδ1 cells recognize lipids presented by CD1 molecules and are enriched in CD1-specific T cells,[33,34] and that CD1–sulphatide-specific cells in MS lesions may be a specialized subset of Vδ1-positive type II NKT cells. Note that while CD1d–sulphatide-specific TCRs express similar Vδ1-Jδ1 chains, they can pair with different Vγ chains.[32] It will be informative to determine whether Vδ1-Jδ1-positive type II NKT cells are pathogenic or regulatory in a demyelinating disease, bearing in mind that $V\delta1^+$ T cells can dominate γδ T-cell populations in the lesions and cerebrospinal fluid of MS patients.[35–37]

Antigen recognition by NKT cell subsets

NKT cells are generally autoreactive and can recognize both exogenous and endogenous lipids. Reactivity of mouse and human NKT cell subsets to common self lipid antigens is shown in Table 2. Type I NKT cells were initially characterized following recognition of α-galactosylceramide (αGalCer), a glycolipid derived from the marine sponge. Notably, αGalCer binds with extraordinarily high binding affinity and stimulates type I NKT cells like a superantigen. Most microbial lipids and other self antigens, including isoglobotrihexosylceramide, or isogloboside 3 (iGB3),[38] do not stimulate type I NKT cells very effectively. Therefore, the *in vivo* effects of αGalCer stimulation may not reflect true physiological responses because of its non-mammalian nature. Further studies are required to identify the underlying biology and mechanisms of type I NKT cell recognition of self antigens. Furthermore, type I NKT cells can also be activated in a CD1d-independent manner by exposure to several cytokines such as IL-12 and IL-18 or IL-12 and type I IFN.[39–41] In addition to αGalCer, several self antigens have been shown to stimulate type I NKT cell activity.[42] Among these antigens, some self lipids including β-D-glucopyranosylceramide (β-GlcCer), lysophosphatidylethanolamine and lysophosphatidic acid are recognized by both mouse and human type I NKT cells. Human but not murine type I NKT cells are also reactive to lysophosphatidylcholine and lysosphingomyelin. Hence, different self antigens can potentially stimulate type I NKT cells, and some of these antigens are present at elevated levels during inflammation.

As mentioned above, one of the first antigens found to be recognized by a major subset of type II NKT cells is a self glycolipid, termed sulphatide. Recently, other self lipids including β-GlcCer and β-GalCer, as well as some pollen-derived lipids, were shown to be recognized by type II NKT cells.[30,43–45] Interestingly, lysophosphatidylethanolamine induced following hepatitis B virus infection may be a self antigen for a subset of type II NKT cells.[46] We recently identified another phospholipid lysophosphatidylcholine to be effective in stimulating type II NKT cells both *in vitro* and *in vivo* (I. Maricic, manu-

script in preparation). Previously, lysophosphatidylcholine was reported to activate human type II NKT cells in lymphomas.[47] These findings identify some redundancy and an overlapping TCR repertoire among type II NKT cells that recognize self lipids. It will be interesting to determine whether most self lipids that activate type I NKT cells differ from or are similar to those that activate type II NKT cells upon antigen presentation *in vivo*. The finding that a number of microbial lipids preferentially activate type I NKT cells begs that the following question be addressed – can a semi-invariant TCR bias the recognition of microbial antigens by type I NKT cells? Future studies using altered lipid ligands and individual mutations in key residues of TCR α and β chains may unravel some of these features of lipid recognition.

Recent insights from the crystal structure of a type II NKT cell TCR that recognizes sulphatide and lysosulphatide suggested the presence of a distinct recognition motif for TCR recognition between the type I and type II NKT cell subsets.[30,48,49] How are these differences in antigen recognition between type I and II NKT cells selected and maintained, and what are the consequences of this differential antigen recognition by these NKT cell subsets in health and in disease? For example, it is clear that type II NKT cells reactive to sulphatide still develop in mice that are deficient in enzymes required for the synthesis of sulphatide.[27,28] Other self lipids may either compensate for the selection of sulphatide-reactive TCR or may not be essential for the development of type II NKT cells. Additional studies are needed to resolve whether self lipids are required for the development of NKT cells in general.

NKT cell recirculation and migration into tissues

Conventional $CD4^+$ T cells

During immune responses, T cells and B cells migrate and recirculate between blood and peripheral lymphoid tissues before activation by antigens. In tissues such as lymph nodes and spleen, T cells are recruited by chemokines to sites of interaction with resident antigen-presenting DCs. Upon subsequent exposure to antigens, T cells proliferate and differentiate into effector T cells (Teff) that migrate to sites of infection to eliminate pathogens. Hence, many lymphocytes at different stages of activation are recruited to different peripheral lymphoid sites to carry out their functions. This process of lymphocyte recirculation is critical for T cells, as Teff cells must target and eliminate pathogens at sites of inflammation or infection.[11] Clearance of infectious pathogens is also dependent on the action of cytokines secreted by Teff. Critical T-cell–DC interactions occur at sites of inflammation in lymph nodes and thereby control susceptibility to the development of an autoimmune disease. Therefore, it is crucial to understand how the dynamics of T-cell recirculation, localization and interaction *in vivo* within tissues such as lymph nodes contribute to effective immune responses that either promote or prevent inflammation and autoimmune disease.

Intravital imaging

Recent application of intravital imaging technology, which uses two-photon (2P) microscopy to detect the location, behaviour, movement and interactions of viable cells *in vivo*, has significantly advanced our understanding of several factors that mediate T-cell–DC and T-cell–B-cell interactions.[50–54] We have learned how such cells behave in resting tissue, how they interact with one another, exchange information, respond to pathogenic stimuli, and mediate various functions. This technique has also been informative about disease processes that occur in cells by defining the impact of specific changes in real-time. Visualization and quantification of these cellular dynamics *in vivo* relies on the ability to fluorescently tag different cell types under analysis. For example, the use of 'photoswitchable' fluorescent proteins that transition from green to red can track individual cells as they move between blood vessels and tissues in the body. Currently, most studies are limited to a tissue depth of about 300–400 mm. Major conclusions reached so far using 2P microscopy of fluorescently tagged cells are summarized in Table 3.

Another conclusion of particular interest is that the duration of T-cell contact with APCs may vary from being long-lived if they occur during an immune response to short-lived while they are in a state of peripheral tolerance. Conceivably, this difference in duration of T-cell–APC contact could be diagnostic of the capacity of various agents administered *in vivo* to treat a given disease to induce (pre-disease onset) or restore (post-disease onset) immune tolerance. In this regard, imaging studies have reported that the inhibitory receptors cytotoxic T-lymphocyte antigen-4 and programmed death-1 on Teff or Treg cells may suppress immune responses by limiting the duration of T-cell interaction with antigen-bearing DCs.[55–57] While intriguing, these results on duration of T-cell–APC contacts remain controversial and may vary depending on the specific experimental systems used.[58–60] It is also controversial as to whether brief contacts between T-cell effectors (e.g. cytokines) and APCs deliver a sufficient quantity of effector molecules to elicit chronic inflammation. Since the delivery of such effector molecules requires long T-cell–APC contacts, it is anticipated that only a relatively small number of effector molecules are secreted at any given time when antigen concentration is limited. Conceivably, under conditions of high antigen concentration, the duration of T-cell–APC contacts is longer and sufficient to elicit a chronic inflammatory response. Hence, it has been suggested that the presence

Table 3. Conclusions from intravital imaging studies of lymphocytes in mouse lymph nodes

Number	Conclusion
1	Lymph nodes are major sites of antigen capture, detection and adaptive immune responses. T cells recruited to lymph nodes can spend a few hours to 1 day sampling APCs for antigen, but they frequently leave the nodes without finding antigen
2	While encounters between T cells and their specific antigens occur randomly, the frequency of such encounters is enhanced by the expression of antigen-specific TCRs on T cells and the ability of most T cells to recirculate through lymph nodes at least once per day. This recirculation permits populations of both naive T cells and memory T cells to scan lymph nodes (and other lymphoid tissues) on a daily basis for the presence of pathogens and eliminate them if necessary
3	Prolonged T-cell–DC contacts arise when several T cells congregate around individual DCs. After 16–24 hr in a lymph node, activated $CD4^+$ T cells resume their motility, pursue several rounds of proliferation, and then interact with cognate B cells located proximal to a follicle
4	The strategic use of photoactivatable probes, which can tag cells present in one location and image them while enroute to another site, have shown that the extent of T-cell–DC interactions in a given zone of a lymph node influences the migration of T cells to another zone(s)
5	Upon migration in a lymph node, B cells may adhere to T cells and drag the T cells behind them. T-cell–B-cell contacts of sufficient time and strength are required to stimulate T-cell signals (e.g. CD40 ligand, cytokines). During recognition of these signals by antigen-activated B cells, T cells depolarize and then adhere to and follow the B cells
6	In response to increased expression of adhesion molecules, chemokines and chemokine receptors in T-cell zones of lymph nodes, naive $CD4^+$ T cells migrate more rapidly than other cell types through high endothelial venules into regions where antigens are displayed by DCs. T cells may spend between a few hours to a day in these regions, and their entry and exit from a lymph node is mediated by the chemokine receptor CCR7 and sphingosine-1-phosphate receptor 1, respectively
7	Local T-cell motility in a lymph node can transform into long-range migrations by T cells that leave this tissue and migrate in blood or lymph to distant sites
8	The duration of contact between T cells with DCs or B cells in a lymph node determines the extent of immune responsiveness. The capacity of regulatory T cells to down-regulate $CD4^+$ T-cell priming and ability of inhibitory receptors on T cells to block $CD8^+$ effector T-cell activity is mediated by the increased surface expression of CTLA-4 and PD-1. By reducing the time of effective cell–cell contact between T cells and antigen-bearing DCs or B cells and the time that cellular receptors remain engaged, the inhibitory effects of CTLA-4 and PD-1 are amplified upon signalling through the TCR or costimulatory molecules

APCs, antigen-presenting cells; CTLA-4, cytotoxic T-lymphocyte antigen 4; DCs, dendritic cells, PD-1, programmed death 1; TCR, T-cell receptor.

of antigen at a relatively low concentration may be protective against inflammation.[54] Further experimentation is required to address this question, as well as the questions of how long are cytokines produced by T cells in antigen-rich versus antigen-poor tissue environments and are effector cytokines retained locally or can they be delivered to several different distant sites.

NKT cells

Similar to the above-described patterns of recirculation and migration of naive, effector and memory $CD4^+$ T cells, recent studies have also analysed the patterns of recirculation and migration of NKT cells *in vivo* in mice (Table 4).[60] The pathogenic and protective effects of NKT cell subsets following agonist stimulation *in vivo* are determined mainly by their timing of activation, structures of lipid antigens recognized, interactions with different DCs and profiles of cytokine secretion. Using structural variants of αGalCer that do not interfere with TCR recognition, it was recently shown that distinct types of CD1d-bearing DCs may regulate the different profiles of cytokines secreted, e.g. Th1-type (IL-12, IFN-γ), Th2-type [IL-4, IL-9, IL-10, IL-13, granulocyte–macrophage colony-stimulating factor (GM-CSF)] or Th17-type (IL-17A, IL-21, IL-22), by NKT cells *in vivo*.[32,60] The list of cytokines secreted by NKT cells include IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, tumour necrosis factor-α, IFN-γ, transforming growth factor-β and GM-CSF. Hence, depending on the type of specific interactions between subsets of NKT cells and DCs, the cytokines secreted by activated NKT cells may either activate or suppress adaptive immune responses. Since the strength of a TCR signal may influence the cytokine profile (Th1- or Th2-type) produced, understanding how the TCRs of NKT cell subsets bind to their ligands and subsequently cross-regulate each other's activity is essential for the development of improved strategies of immune regulation for intervention in autoimmune diseases (Table 5). Considerable recent evidence in favour of a regulatory function of both type I and type II NKT cells suggests that both NKT cell subsets are attractive targets to test in novel immunotherapeutic protocols.[7–14,61–63]

A valuable animal model in which to study the pattern of recirculation and migration of NKT cells *in vivo* is a mouse in which the green fluorescent protein (GFP) gene is

Table 4. Conclusions from intravital imaging studies of mouse natural killer T (NKT) cells

Number	Conclusion
1	Cxcr6$^{gfp/+}$ mice are knock-in mice that have green fluorescent protein integrated into the *Cxcr6* chemokine receptor gene
2	In Cxcr6$^{gfp/+}$ mice, type I NKT cells migrate to the liver, where they are most abundant (20–30% of lymphocytes). This migration is arrested in liver sinusoids upon encounter with antigen presented on sinusoidal epithelial cells within minutes after injection of αGalCer
3	Inflammatory cytokines, such as IL-12 and IL-18, that can activate type I NKT cells also induce an arrest in motility of type I NKT cells in liver sinusoids of Cxcr6$^{gfp/+}$ mice without the need for CD1d antigen presentation. This cytokine-induced arrest is rapid (occurs within 1 hr), precedes NKT cell activation, and upon antigen encounter stabilizes the formation of an immune synapse between NKT cells and interacting APCs. The latter interactions trigger the secretion of IL-4 and IFN-γ by sinusoidal type I NKT cells
4	Due to constitutive interactions between the adhesion molecules LFA-1 and ICAM-1 that result from this NKT–APC synapse formation, activated type I NKT cells are retained in the liver and recirculate less than activated CD4$^+$ T cells. Hence, LFA-1 and ICAM-1 function to retain type I NKT cells in the liver
5	After a stroke, type I NKT cells rapidly exit the liver and elicit bacteraemia. In addition, in the lung of αGalCer-treated mice, NKT cells extravasate from the lung and trigger inflammation and adaptive immune responses. Hence, the patterns and kinetics of recirculation of type I mouse NKT cells differ in a tissue- and stimulus-dependent manner
6	Type I NKT cells can recognize bacterial ligands (e.g. α-galactosyldiacylglycerols of *Borrelia burgdoferi*) and contribute to the prevention and clearance of bacterial infections

APCs, antigen-presenting cells; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; IL-12, interleukin-12; LFA-1, lymphocyte function-associated antigen 1.

knocked into a lineage-specific gene yielding a heterozygous mouse in which certain leucocytes are fluorescently labelled.[61] The salient features of NKT cell recirculation and migration obtained in such a mouse model are highlighted in Table 4. In knock-in mice that have GFP integrated into the *Cxcr6* chemokine receptor gene (Cxcr6$^{gfp/+}$ mice), type I NKT cells migrate to the liver where they are quite abundant (20–30% of lymphocytes). This NKT cell migration *in vivo* is arrested in liver sinusoids upon encounter with antigen presented on sinusoidal epithelial cells within minutes after injection of αGalCer.[41,64–67] In addition to antigen, the IL-12 and IL-18 pro-inflammatory cytokines also terminate type I NKT cell motility in liver sinusoids of Cxcr6$^{gfp/+}$ mice in a CD1d-independent manner. The latter arrest in NKT cell movement occurs by 1 hr after exposure to the cytokines and precedes NKT cell activation. Subsequent antigen encounter stabilizes the formation of an immune synapse between NKT cells and interacting APCs. This synapse elicits lymphocyte function-associated-1/intercellular adhesion molecule-1 interactions that enable activated type I NKT cells to be retained in the liver, demonstrating that activated type I NKT cells recirculate less than activated conventional CD4$^+$ T cells.[68] However, after a stroke, type I NKT cells rapidly exit the liver and elicit bacteraemia. Similarly, NKT cells extravasate rapidly from the lung of αGalCer-treated mice and trigger inflammation and adaptive immune responses.[69] Hence, the patterns and kinetics of recirculation of type I mouse NKT cells differ in a tissue- and stimulus-dependent manner. Additional studies are required to unravel the mechanisms involved and to determine whether this variation in recirculation exists for mouse type II NKT cells and human type I and type II NKT cells.

NKT cells in autoimmune disease in humans

Humans possess both CD4$^+$ and CD4$^-$ type I NKT cells.[11] Although both subsets secrete Th1-type cytokines, CD4$^+$ type I NKT cells secrete predominantly Th2-type cytokines. In a population of Th1-like CD4$^-$ NKT cells, CD8α^+ cells comprise a large subset and CD8$\alpha\beta^+$ cells a small subset. CD8α^+ type*I*NKT cells secrete more IFN-γ and possess greater cytotoxic activity than do CD4$^+$ or CD4$^-$ NKT cells. In human peripheral blood, type I NKT cells comprise about 0·1–0·2% of T cells, but this proportion is highly variable and can range from $< 0{\cdot}1\%$ to $> 2\%$.[70–72] Twin studies suggest that the number of human type I NKT cells in PBMCs is genetically regulated.[4] Interestingly, human type I NKT cells are enriched in the omentum (about 10% of T cells) and not in the liver.[73,74]

Reduced numbers of type I NKT cells in PBMCs appear to correlate with several autoimmune or inflammatory conditions and cancers,[75] but this finding remains controversial. Similarly in patients with rheumatoid arthritis, PBMCs[76,77] and synovia[78] display lower levels of NKT cells as well as a Th1 bias during disease.[77] Interestingly, patients with myasthenia gravis display elevated levels of type I NKT cells in PBMCs, in contrast to those in PBMCs from patients with MS,[75] rheumatoid arthritis[76] and type 1 diabetes[79]. The reason for these differences is currently unknown. Nevertheless, NKT cell levels return to normal levels after treatment.[80] Moreover, the number of type I NKT cells in the blood and lung of patients with sickle cell disease is significantly increased.[81,82] The reasons for this reduction and increase, respectively, are not known, but may be linked in part to differences in the

Table 5. Role of natural killer T (NKT) cells in susceptibility to autoimmune disease in mice

Disease	NKT-deficient ($CD1^{-/-}$ $J\alpha18^{-/-}$)	Activation of type I NKT cells (αGalCer)	Activation of type II NKT cells (Sulphatide)
Spontaneously developed			
Type 1 diabetes	disease	protection	protection
Systemic lupus erythematosus	disease	disease or protection	protection
Antigen-induced			
Experimental allergic encephalomyelitis	same or less	disease or protection	protection
Collagen-induced arthritis	less	protection	–
Experimental allergic myasthenia gravis	same or less	protection	–
Primary biliary cirrhosis	same or less	protection	–
Experimental allergic uveitis	same or less	protection	–
Hepatitis	less	disease	protection

patterns of motility and recirculation of different NKT cells in the blood and target tissues in these and other diseases. In future studies, it will be important to determine whether healthy individuals with a diminished NKT cell frequency in blood and target tissues are at a higher risk for disease. This will require longitudinal studies in cohorts of sufficient size and statistical power, but may prove problematic because it is uncertain whether the frequency of NKT cells in PBMCs accurately reflects the size and frequency of systemic or organ-specific NKT cell pools in humans.[75]

Hence, other approaches may be more informative about the role of NKT cells in human diseases. First, it is possible that NKT cell defects are caused by polymorphisms in molecules that are essential for NKT development, such as the signalling lymphocyte activation molecule[83] and promyelocytic leukaemia zinc finger[84] pathways. If so, genetic assays of these polymorphisms should be performed routinely in various human conditions. Second, longitudinal analysis in humans with a particular disease is essential for observing changes in NKT cell number and cytokine secretion patterns during disease progression[75] to assess their possible role. Correlation of the frequency of NKT cells with their cytokine patterns and disease onset will probably enhance our understanding of the aetiology of an autoimmune disease.[2–14]

To further determine the various properties of human NKT cells in health and disease, analyses of migration and recirculation of human NKT cell subsets *in vivo* in animal models may help us to better understand the biology and mechanisms of cellular interaction of human NKT cell subsets with APCs. Two such animal models are available. First, the high level of expression of CXCR6 by human NKT cells enables the use of the $Cxcr6^{gfp/+}$ mice described above to study the dynamics of movement, positioning and activation of human NKT cells *in vivo*. Second, the cellular dynamics of human CD1d (hCD1d) -restricted NKT cells may be monitored in hCD1d knock-in mice in which the expression of murine CD1d is replaced by hCD1d.[85] These mice harbour a subpopulation of type I NKT cells that resemble human type I NKT cells in their tissue distribution, phenotype (express mouse Vβ8, a human Vβ11 homologue, and low levels of CD4) and function (antitumour activity). It is anticipated that humanized hCD1d knock-in mice will permit the *in vivo* modelling of lipid antigen-induced migration and function of hCD1d-restricted type I, and possibly type II, NKT cells. Hence, such studies may facilitate the evaluation of novel drugs targeted *in vivo* for type I and type II NKT cell therapies in humans.

A detailed characterization of type II NKT cells and their ligands in humans is required for their appropriate manipulation in disease conditions, as was recently initiated for the antitumour effects of type I NKT cells in phase I/II clinical trials with αGalCer in humans.[85–88] Other analogues of αGalCer that are able to skew conventional $CD4^+$ T-cell responses more towards either a Th1- or a Th2-like profile will be introduced into clinical studies. In the near future, it may be possible to differentially activate or inhibit type I and type II NKT cells for the development of novel immunotherapeutic protocols in the treatment and prevention of autoimmune diseases.

Immune mechanisms following NKT cell activation

Mechanisms by which NKT cell subsets modulate immunity generally follow events and their interactions with other immune cells after activation by their respective lipid antigens, e.g. αGalCer and sulphatide for type I and type II NKT cell subsets, respectively. As DCs play a crucial role not only in the activation of NKT cells but also may be central to their role in the regulation of immune responses, we will first consider NKT–DC interactions and their control of NKT cell-mediated modulation of autoimmune disease.

NKT cell subsets and DC interactions

The advent of intravital imaging now enables the cell dynamics and function of T-cell–DC interactions to be investigated *in vivo*. Considerable new information provided by the application of 2P microscopy has been reported about the cellular and molecular dynamics of conventional $CD4^+$ and $CD8^+$ T-cell–DC interactions *in vivo*.[51,54] While NKT–DC interactions are also central to the regulation of many immune responses and diseases, less is currently known about the dynamics of movement, recirculation and interaction between NKT cells and DCs *in vivo*.[51,54] Some recent observations made using *in vivo* imaging of NKT–DC interactions are presented in Table 6. A key finding is that bidirectional NKT cell–DC interactions can elicit and amplify innate and adaptive immune responses. Hence, intravital imaging has identified a central role for NKT cells in the context of other immune cells during various immune responses.[51,54] This further underscores the importance of learning more about different NKT cell subsets and developing more experimental approaches to track these NKT cell subsets by *in vivo* imaging.

Table 6. Properties of natural killer T (NKT) cell–dendritic cell (DC) interactions

Number	Property
1	DCs are the major interacting APCs that activate type I NKT cells after exposure to lipid antigens *in vivo*
2	In local draining lymph nodes, DCs display lipid antigens and stimulate interacting type I NKT cells to secrete IFN-γ. The targeting of lipid antigens to various DCs depends critically on lipid structure and can alter the biological response elicited
3	Interactions between I NKT cells and DCs are bidirectional
4	During infection, pathogen-derived signals stimulate DCs to produce IL-12 and enhance their production of lipid antigens that stimulate type I NKT cells. These activated NKT cells then recognize self or foreign lipid antigens presented by CD1d on DCs. Upon ligation of CD40 to CD40 ligand, the NKT cells in turn induce further IL-12 production by DCs, which binds to IL-12R on NKT cells and activates them. These DC–NKT cell interactions can activate NK cells, enhance $CD4^+$ and $CD8^+$ T-cell responses to protein antigens and facilitate DC cross-presentation
5	This ability of activated type I NKT cells to function centrally in the stimulation of adaptive T-cell responses has important clinical implications. For example, αGalCer is an efficient vaccine adjuvant that can generate protective T-cell immunity to co-administered protein antigens

APCs, antigen-presenting cells; IFN-γ, interferon-γ; IL-12, interleukin-12; iNKT, invariant natural killer T.

In such studies, it is essential to monitor before and after antigen stimulation: (i) the tracking patterns of type I and type II NKT cells from blood into peripheral tissues (e.g. lymph nodes, spleen, liver), (ii) the differences in the number, time and stability of encounters of these NKT subsets with DCs, (iii) the time and sites of migration of these subsets after DC interaction, and (iv) these various parameters in environments of health (e.g. normal disease-free mouse strains) or disease (e.g. mouse strains that develop different autoimmune diseases, as described below). The ligand antigens to be administered to the mice to stimulate the motility and immune responses of their type I and type II NKT cells could initially be α-GalCer and sulphatide, respectively. While α-GalCer activates type I NKT cells specifically, sulphatide is recognized only by type II NKT cells. *In vivo*, type I NKT cells could be tagged and tracked by staining with fluorescently labelled α-GalCer/CD1d tetramers, as reported.[89] We have shown that in non-obese diabetic (NOD) mice that spontaneously develop type 1 diabetes, both type I and type II NKT cells accumulate in draining pancreatic lymph nodes. Moreover, treatment of NOD mice with sulphatide C24:0 (long isoform) protects them from type 1 diabetes more efficiently than does treatment with sulphatide C16:0 (short isoform). Our data suggest that sulphatide C24:0 stimulated type II NKT cells may regulate protection from type 1 diabetes by activating DCs to secrete IL-10 and suppress the activation and expansion of type I NKT cells and diabetogenic $CD4^+$ and $CD8^+$ T cells.[89] Imaging of the cellular dynamics and motility of type I and type II NKT cells, as well as their interactions with DCs, in NOD mice treated with sulphatide C24:0 or sulphatide C16:0 would allow us to further test the proposed roles of these NKT cell subsets in protection from experimental type 1 diabetes. Since Treg cells are needed to help activated type I NKT cells protect NOD mice from type 1 diabetes,[90] the relative role of Treg cell–DC interactions in protection from type 1 diabetes could also be monitored using laser-induced photoactivatable fluorescent protein probes to label Treg cells in a defined location (e.g. pancreatic lymph node) and to then track their movement and fate over time.[51] It will also be interesting to compare the location, time and strength of interactions between DCs and either islet autoantigen-specific $CD4^+$ T cells, type I or type II NKT cells, or Treg cells in lymph nodes both in the pancreas and in other anatomical sites. Whether these various T-cell subsets resume their motility, swarm in the local vicinity and undergo proliferation following DC encounters will prove informative about the relative contributions of NKT subsets and Treg cells in protection from type 1 diabetes. Finally, to better comprehend how intracellular signalling influences communication between T cells and DCs *in vivo*, the role of calcium signalling (see below) during either type I NKT cell, type II NKT

cell or Treg cell migration and activation could be followed using intracellular dyes that change fluorescence upon binding to calcium.[51]

Type I and type II NKT cell anergy

Several studies have shown that after chronic stimulation by αGalCer as well as cross-regulation induced by type II NKT activation, type I NKT cells can be anergized. *In vivo* imaging analyses may reveal novel features about the regulation of anergy induction in type I NKT cells, as exemplified in three experimental mouse models. In the first model, the C20:2 N-acyl variant of αGalCer, a Th2-biasing derivative of αGalCer, was shown to activate type I NKT cells in NOD mice more weakly than αGalCer. Type I NKT cells activated *in vivo* with C20:2 enter into and exit from anergy more rapidly than after activation by αGalCer.[91,92] This C20:2 induced shorter duration of type I NKT cells in the anergic state promotes the more rapid induction of tolerogenic DCs in an IL-10-dependent manner, gives rise to reduced type I NKT cell death, and enables C20:2-stimulated type I NKT cells to elicit enhanced protection from type 1 diabetes. These findings suggest that C20:2 may be more effective for disease intervention than αGalCer for protection from type 1 diabetes. It is anticipated that further support for this possibility could be obtained by more informative *in vivo* imaging studies of the dynamics and kinetics of interaction between type I NKT cells and DCs in pancreatic lymph nodes of NOD mice treated *in vivo* with either αGalCer or C20:2. In addition, 2P imaging *in vivo* of differentially activated and anergic NKT cells will further elucidate how a short versus long duration of NKT cell anergy can regulate poor versus strong protection from type 1 diabetes.

In a second model, 2P imaging may offer more insight into whether C24:0 sulphatide activates type II NKT cells to enter into and exit from anergy more rapidly than C16:0 sulphatide activation and thereby yield less type II NKT cell death and increased protection from T1D.[89] Finally, a third model is based on the report that activation of sulphatide-reactive type II NKT cells and DCs elicits the IL-12- and macrophage inflammatory protein 2-dependent recruitment of type I NKT cells into the liver.[62] The latter recruited type I NKT cells are anergic and prevent concanavalin A (Con A) -induced hepatitis by specifically blocking effector pathways, including the cytokine burst and neutrophil recruitment following Con A injection. Hepatic DCs from IL-$12^{+/+}$ but not from IL-$12^{-/-}$ mice can adoptively transfer type I NKT cell anergy into recipient mice. Hence, IL-12 secretion by DCs enables them to induce anergy in type I NKT cells. These data describe a novel mechanism by which type II NKT cell–DC interactions in the liver can cross-regulate the activity of type I NKT cells. Further *in vivo* imaging analyses may help to demonstrate whether this type of immune cross-regulation applies to human NKT cell subsets. If this is the case, such studies may facilitate immune intervention in inflammatory and autommmune diseases in humans.

Ca^{2+} signalling

The ability to detect intracellular signalling that occurs during T-cell–DC contacts by 2P imaging *in vivo* has dramatically improved our understanding of cellular communication during immune responses.[51,54] While a brief contact of T cells with antigen-bearing DCs induces T cells to pause momentarily and then continue their migration, these T-cell–DC interactions also induce Ca^{2+} signalling in T cells that promptly reduces T-cell motility. The Ca^{2+} signals may synergize with other signalling pathways to stimulate T-cell gene expression, cytokine secretion and proliferation. Interestingly, the strength of Ca^{2+} signaling in T cells depends on the time of direct contact of T cells with DCs. After initial T-cell–DC contacts, T cells migrate again and sample several other DCs. However, T-cell migration is diminished appreciably in the presence of an antigen with high affinity for a given TCR that elicits a relatively strong Ca^{2+} signal in T cells. The continued use of intracellular dyes that change their fluorescence properties upon binding to Ca^{2+} will advance our investigation of this crucial role of Ca^{2+} signalling in T-cell migration and antigen recognition. Hence, 2P microscopy coupled with the quantification of intracellular Ca^{2+} signalling by T cells activated by different antigens *in vivo* can be informative about the relative strength of T-cell–DC interactions and the immune responses that follow under conditions of health and disease.

The relative strength of TCR signalling *in vivo* can also be measured by following the shedding of CD62L from the surface of T cells.[93] A few minutes after TCR activation in a T cell, the CD62L extracellular domain is cleaved by the protease ADAM17 (a disintegrin and metalloproteinase domain-containing protein 17). The extent of CD62L shedding reflects TCR signal strength, i.e. a strong TCR signal elicits increased shedding of CD62L. Hence, T-cell dynamics *in vivo* may be tracked together with TCR signals by measuring the disappearance of CD62L after *in vivo* staining with fluorescent anti-CD62 antibody Fab fragments.

Influence of NKT cell subsets in experimental autoimmune disease

Physiological role of NKT cells

The functional role of NKT cells has been analysed in mice using CD1d$^{-/-}$ (lack both type I and type II NKT

cells) and J$\alpha18^{-/-}$ (lack only type I NKT cells) mice as well as using blocking or depleting antibodies reactive to CD1d and the semi-invariant TCR. The combined use of both of these mouse strains and antibodies has allowed us to ascribe the outcome of specific immune responses to the effect of either type I NKT cells or type II NKT cells. However, various compensating mechanisms, such as an altered conventional TCR repertoire, may control NKT cell function in such knockout mouse environments. Our understanding of the roles of NKT cells in the induction and/or protection from autoimmune disease has taken advantage of analyses of NKT cells in such diseases that either arise spontaneously or are antigen-induced (Table 4). It is important to note while αGalCer has been informative about type I NKT cell activation and function, it has not revealed a comprehensive understanding of the physiological role of type I NKT cells.

A role for type I NKT cells in the regulation of autoimmune disease was provided by observations that fewer type I NKT cells are found in both spontaneous autoimmune disease models, type 1 diabetes in NOD mice and systemic lupus erythematosus in MRL/lpr mice.[94,95] However, CD1d deficiency did not result in potentiation of disease, as expected in all models.[96–100] In the case of lupus, one study indicated that CD1d$^{-/-}$ mice do not develop renal disease or skin lesions,[101] whereas other studies have suggested that CD1d$^{-/-}$ mice express an exacerbated lupus skin disorder but not renal symptoms.[95] Furthermore type I NKT cells in spontaneous disease in (NZB × NZW F_1) mice, were shown to promote anti-dsDNA autoantibody production by B cells *in vitro* as well as *in vivo* following adoptive transfer.[102–107] However, in NOD mice, spontaneous diabetes was exacerbated in CD1d-deficient animals lacking both NKT cell subsets. Hence, the physiological role of type I NKT cells remains controversial in spontaneous autoimmune diseases, possibly due to the absence of both NKT cell subsets in CD1d$^{-/-}$ mice as well as differences in background genes, alterations in the target tissues and site(s) of priming of NKT cells.

Role of type I NKT cells in antigen-induced autoimmune disease

It is important to note that in most autoimmune disease models antigens or peptides are administered following their emulsification in complete Freund's adjuvant. It is clear that type I NKT cells have an adjuvant-like effect, especially upon activation with αGalCer and can stimulate the activation of DCs. Therefore, the physiological contribution of type I NKT cells in experimental autoimmunity may be compromised, particularly if αGalCer is administered at the time of antigen/complete Freund's adjuvant administration as it can potentiate Th1 ell-mediated diseases.[108–111] Similarly, αGalCer administration can bias a global Th-dependent response towards a Th1-like or Th2-like polarized response. For example, continuous αGalCer injection in younger (4-week-old) lupus-prone mice partially alleviates systemic lupus erythmatosus symptoms by increasing a Th2 bias,[112] whereas identical treatment in older mice (8–12 weeks old) increases a Th1-biased cytokine secretion profile and disease severity.[108]

In most experimental autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune myasthenia gravis,[19,91,112–115] antigen-induced disease is generally either less severe or not affected in CD1d$^{-/-}$ or J$\alpha18^{-/-}$ mice. These data suggest that type I NKT cells may help in the priming of antigen-reactive T cells by activating conventional DCs and may not be regulatory in this context. These data also indicate that induction of antigen-induced autoimmune disease is not dependent upon the presence of type I or type II NKT cells. Rather, as a result of the administration of complete Freund's adjuvant, type I NKT cells may elicit an adjuvant-like effect and thereby contribute to the severity of disease by potentiating Th1/Th17-like responses.[19] Consistent with this view, a general skewing of a conventional Th cell response towards a Th2-like cell response by αGalCer or its analogues, e.g. OCH, leads to protection from some autoimmune diseases, including EAE, type 1 diabetes, and collagen-induced arthritis.[47,80,91,94,113–117] Interestingly, in some cases, an IFN-γ bias can also protect from EAE and experimental autoimmune uveitis by inducing the apoptosis of pathogenic CD4$^+$ T cells.[80] Consistent with the other disease models, in the case of primary biliary cirrhosis, J$\alpha18^{-/-}$ mice are resistant to disease induction and CD1d$^{-/-}$ mice have a reduced incidence of cholangitis, indicating a pathogenic role for type I NKT cells.[118,119] Similar to some of the EAE models, stimulation of type I NKT cells with αGalCer results in disease exacerbation associated with a Th1 cytokine release profile.[118–121] In the latter cases, type I NKT cell activation by αGalCer or its analogues may lead to the tolerization of APC populations. In turn, this outcome may inhibit the activity of most Th1/Th17/Th2 secreting effector cells and thereby lead to protection from autoimmune disease.

Role of type II NKT cells in antigen-induced autoimmune disease

Generally, activation of type II NKT cells with self-glycolipid sulphatide may control both antigen-induced and spontaneously arising autoimmune disease. During EAE, sulphatide-reactive type II NKT cells, but not type I NKT cells, are increased several fold in the CNS. This greater abundance of type II NKT cells in the CNS inverts the usual ratio of type II : type I NKT cells (type II NKT cells, 3–4%; and type I NKT cells, 0·6–0·9%) and affords protection from EAE.[27,61] Furthermore, administration of

sulphatide to activate type II NKT cells decreases the number of IFN-γ- and IL-17-secreting myelin basic protein and proteolipid protein-reactive encephalitogenic $CD4^+$ T cells. The net outcome is protection from EAE via a CD1d-dependent regulatory pathway (Maricic *et al.*, submitted). This type II NKT-mediated immunoregulatory pathway results in (i) inactivation of type I NKT cells that now function as regulatory T cells, (ii) tolerization of conventional DCs, (iii) tolerization of microglia in the CNS and (iv) inhibition of the effector functions of pathogenic MHC-restricted $CD4^+$ T cells. As APCs that activate pathogenic Th1 and Th17 cells in lymphoid organs and the CNS are tolerized following sulphatide administration, activation of type II NKT cells induced by sulphatide is much more potent in the regulation of autoimmune demyelination than only the inactivation of type I NKT cells by αGalCer (Maricic *et al.*, submitted).

Activation of type II NKT cells by sulphatide was recently reported to protect NOD mice from type 1 diabetes.[28,89] Pre-treatment of NOD mice with the C24:0 but not C18:0 sulphatide analogue was found to protect against the transfer of type 1 diabetes.[89] These data suggest that the longer C24:0 sulphatide analogue should be examined for its therapeutic value in clinical trials in human subjects at risk for or newly diagnosed with type 1 diabetes. Our preliminary studies suggest that activation of type II NKT cells following administration of sulphatide significantly prevents lupus nephritis in (NZB × NZW) F_1 mice, indicating that the protective capacity of sulphatide activated type II NKT cells can counteract potentially pathogenic type I NKT cells.

Gut microbiota may influence the role of NKT cell subsets in autoimmune disease

As both type I and type II NKT cells and several types of $CD1d^+$ cells localize to the intestine, it has been proposed that NKT-$CD1d^+$ cell interactions may be influenced by pathogenic as well as non-pathogenic commensal microbes. Although type I NKT cells seem to recognize lipids of symbiotic commensal bacteria,[120–122] the nature of microbial lipids that activate type II NKT cells is not yet known. Recent findings suggest that both pathogenic and non-pathogenic microbes may modulate intestinal immune responses in healthy and diseased conditions. Evidence from several animal models of experimental inflammatory bowel disease demonstrates that type I NKT cells can be both protective and pathogenic in inflammatory bowel disease.[9] In contrast, type II NKT cells seem to promote intestinal inflammation and may be pathogenic in inflammatory bowel disease when both CD1d expression and the frequency of type II NKT cells are increased in mice as well as patients with ulcerative colitis. However, adoptive transfer studies need to be carried out to substantiate these effects and cross-regulation of NKT cell subsets may further influence the disease outcomes at these sites.

Cross-immunoregulatory pathway of NKT cell subsets

As mentioned above, activation of type II NKT cells with self-glycolipid sulphatide induces a novel regulatory mechanism that may protect from autoimmune disease and inflammatory tissue damage. This unique pathway involves cross-regulation of type I NKT cells and inhibition of pathogenic Th1/Th17 cells through tolerization of conventional DCs (cDCs). It has been shown to be effective in the control of EAE[19,98,109–112], type 1 diabetes,[89] liver diseases,[19,62] and systemic lupus erythematosus (R. Halder, unpublished data). Interestingly, while activation of type I NKT cells predominantly activates hepatic cDCs, sulphatide-mediated activation of type II NKT cells predominantly activates hepatic plasmacytoid DCs (pDCs). Additionally, type II NKT–DC interactions result in a rapid (within hours) recruitment of type I NKT cells into liver in an IL-12 and macrophage inflammatory protein 2-dependent fashion. However, recruited type I NKT cells are neither activated nor secrete cytokines, and consequently become anergic. Hence, anergy in type I NKT cells leads to reduced levels of IFN-γ followed by reduced recruitment of myeloid cells and NK cells and protection from liver damage.[123] Furthermore, tolerized cDCs further inhibit conventional pathogenic $CD4^+$ effector T cells that can elicit autoimmunity.[27] Hence, adoptive transfer of cDCs from sulphatide-treated but not control-treated mice into naive recipients leads to protection against inflammation. Furthermore, activation of sulphatide-reactive type II NKT cells leads to the tolerization of tissue-resident APCs, such as microglia in the CNS. Importantly, this tolerization impairs the development of pathogenic Th1 and Th17 cells.[27] A recent study has suggested that the inducible T-cell co-stimulator and programmed death-1 ligand pathways are required for regulation of type 1 diabetes in NOD mice by $CD4^+$ type II NKT cells.[124] Interestingly, a similar pathway is also involved in regulation by Treg cells.[125,126] Since activation of sulphatide reactive type II NKT cells inhibits the effector functions of pathogenic conventional Th1/Th17 cells in peripheral organs as well as in affected tissues such as the CNS and liver, the targeting of these cells leads to a broader therapeutic response than the targeting of type I NKT cells alone for intervention in autoimmune disease.

Although some studies suggest that type I NKT cells may cross-regulate type II NKT cell activity,[127] additional studies are needed to clarify the mechanisms of regulation involved. It is clear that activation of type I NKT cells with αGalCer leads to a cascade of events that modulates the activity of several cell types, including DCs, B cells, NK cells and neutrophils.[2,3,128] It is likely that sulphatide-mediated induction of anergy in type I NKT cells also

modulates the activity of these other cell types. As mentioned above, our data clearly indicate a significant alteration in the activity of DC populations following sulphatide-mediated activation of type II NKT cells. Current studies are investigating the roles of other cell types that are stimulated after type II NKT cell activation in the presence and absence of type I NKT cells.

Inhibition of effector function or cytokine profile changes in MHC-restricted T cells

Immune regulatory activity of NKT cells can be mediated by the cytokines secreted by NKT cells themselves or following their interaction with other immune cells, including DCs, Treg cells, monocytes and B cells. Hence, activation of NKT cell subsets can result in the deviation of a cytokine secretion profile in MHC-restricted $CD4^+$ T cells towards either a pronounced Th1- or Th2-like response. Generally, for experimental diseases in which Th1 or Th17 cells mediate pathology, immune deviation of the pathogenic T-cell response towards a Th2-like phenotype following type I NKT cell activation with αGalCer or its analogues is protective from disease. For example, protection from type 1 diabetes by NKT cells is associated with an elevated Th2 cytokine profile in pathogenic islet protein-reactive $CD4^+$ T cells,[4,129,130] whereas a Th1 bias correlates with disease severity.[3,109] In spite of this finding, a Th1 to Th2 cytokine profile shift in conventional $CD4^+$ T cells alone may not be sufficient to prevent type 1 diabetes in NOD mice[71,131] or EAE in susceptible mice.[19,98,109–112]

Analyses of cytokine profiles secreted by both activated NKT cells and different APCs after their encounters *in vivo* will also expand our growing knowledge of the mechanisms of leucocyte communication, as described above. Some important questions to be addressed are: (i) how does the duration of specific antigen-induced NKT cell-DC or NKT cell-other APC contacts affect the type and quantity of cytokines and chemokines secreted during pro-inflammatory versus disease-protective immune responses, (ii) how long are cytokines and chemokines secreted by NKT cells in antigen-rich versus antigen-poor tissue environments, and are cytokines and chemokines retained locally or are they delivered to other distant sites to carry out their function, (iii) do the cytokines, chemokines and their respective receptors expressed by interacting NKT cells, DCs and other APCs (hepatocytes or adipocytes) differ under conditions of NKT cell activation, apoptosis and anergy, and (iv) as type I NKT cells are not homogeneous but are comprised of cells with distinct phenotypes and cytokine secretion patterns, e.g. double-negative cells that mainly produce Th1 cytokines, why do these cells become differentially localized in different tissues and how are they activated at these sites.

To address these questions, a technology is required that can track many gene products simultaneously in a viable single cell to resolve any differences between cell subsets (e.g. type I and type II NKT cells) and to define their function in the host. Recently, a new fluorescence single-cell technology was developed that couples flow cytometry with mass spectrometry, and is termed mass cytometry.[132] Mass cytometry permits single-cell analysis of at least 45 simultaneous parameters without the use of fluorochromes or spectral overlap. In this technology, stable non-radioactive isotopes of non-biological rare earth metals are used as reporters to tag antibodies that may be quantified in a mass spectrophotometer detection system. By applying the resolution, sensitivity and dynamic range of this detection system on a timescale that permits the measurement of 1000 cells/s, this methodology offers a new approach to high-content cytometric analysis. For example, the concomitant analysis of expression of cytokines, chemokines and their receptors by mass spectrometry now permits measurement of > 36 proteins/cell at a rate of 1000 cells/s.[133] Similarly, mass cytometry may also be applied to cellular immunophenotyping, which can be used to identify cells based on their surface expression of different antigens or markers. Predictably, further development of flow cytometry and mass cytometry techniques coupled with that of advances in next generation *in vivo* imaging will provide major mechanistic insight in several areas of clinical medicine, including discovery, pathophysiology and therapy of disease.

Role of $CD4^+$ Treg cells and myeloid-derived suppressor cells

Activation of type I NKT cells by αGalCer or its analogues can engage both $FoxP3^+$ $CD4^+$ Treg cells and myeloid-derived suppressor cells in subsequent responses. Cooperation between $CD4^+$ $CD25^+$ Treg cells and type I NKT cells were first shown in experimental models of myasthenia and type 1 diabetes upon activation by αGalCer.[90,114] This protection was primarily mediated by enhanced IL-2 production leading to Treg cell augmentation and inhibition of MHC-restricted T cells. Interestingly, a relationship between type I NKT cells and myeloid cells ($CD11b^+$ $Gr1^+$) cells was initially noted in inflammatory models of liver injury.[124] Following ischaemic reperfusion injury, type I NKT cells are activated, secrete pro-inflammatory cytokines and recruit $CD11b^+$ $Gr1^+$ cells into the liver.[124] Myeloid cells have also been shown to regulate susceptibility to EAE following activation of type I NKT cells by αGalCer.[134] Hence, depletion of immunosuppressive myeloid-derived suppressor cells from the spleen results in the loss of αGalCer-induced protection from EAE. These reports suggest that activation of NKT cell subsets in different tissues may not only lead to their interaction with professional

APCs but also with other immune regulatory cells, including myeloid-derived suppressor cells and Treg cells, and that they can cooperate to provide protection from autoimmune pathology.

Future studies and challenges

In this review, we have attempted to identify key outstanding issues related to the role of NKT cell subsets in health and disease, and how some of these issues may be addressed experimentally and clinically. Based on current evidence, we have proposed a hypothesis that states that while type I NKT cells have pathogenic and protective roles in autoimmune disease susceptibility, type II NKT cells possess mainly a protective role. We have discussed how new experimental mouse models coupled with the application of novel techniques, namely intravital cellular imaging *in vivo* and mass cytometry, may test this hypothesis and also provide important insights into the role of NKT–DC interactions and cytokine/chemokine secretion profiles in determining the outcome of health versus disease.

As the CD1d-dependent antigen recognition pathway is highly conserved from mice to humans, several key features of NKT cell subsets are shared between them. Although most studies in mice have analysed NKT cells from the thymus, spleen or liver, the systemic results of their manipulation indicate that follow-up clinical studies are warranted. Therefore, discoveries in experimental models can be translated into the clinical setting,[1,128] and allow the application of murine studies to humans. Fortunately, type II NKT cells occur more frequently than type I NKT cells in humans, which facilitates their further characterization using appropriate lipid ligands. A detailed characterization of type II NKT cells and their ligands in humans is important for their appropriate manipulation in disease conditions. Phase I/II clinical trials of the anti-tumour effects of human type I NKT cells stimulated by αGalCer have yielded promising results.[71,129–131] Other analogues of αGalCer that skew conventional $CD4^+$ T-cell responses towards either a Th1- or a Th2-like profile remain to be tested in similar trials. In the near future, it may be possible to differentially activate or inhibit type I and type II NKT cells for the development of novel immunotherapeutic protocols in the treatment and prevention of autoimmune disease.

Advances in cellular imaging techniques have provided much recent excitement by enabling the tracking of different cells of the immune system in the blood and peripheral lymphoid tissues *in vivo* in real time. However, these techniques remain limited in their ability to analyse cell motility and interactions (e.g. between NKT cells and DCs) over extended time and distances in intact tissue, and to distinguish between individual cells in a labelled cell aggregate. As stated by Dr Ron Germain, 'the most significant advance currently undergoing development in intravital imaging of the immune system is the combination of molecular imaging with measurements of the dynamics of single cells'.[54] The long-term goal is to attribute cellular movement and positioning to causal changes in cell signalling and gene expression *in vivo*. To achieve this goal, improvements in cell imaging are required and may include increases in the number of different colours used, tissue volume examined and number of cells imaged, duration of imaging sessions, and use of subcellular probes.[51,54] The successful application of these novel technologies will depend largely on the development of new computer algorithms to analyse complex data sets of system biology approaches, including computer simulations.[135,136] Additional studies may benefit from the imaging of higher quality sample preparations from less well-characterized tissues (e.g. gastrointestinal tract, pancreas, spleen and lung). Most importantly, it is envisaged that better diagnostic procedures be achieved in the clinic by introducing miniaturized imaging instruments and light delivery systems in endoscopes or implantable devices.[54]

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Disclosures

The authors declare no conflict of interest.

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