

OPINION

Natural killer T cells: drivers or passengers in preventing human disease?

Stuart P. Berzins and David S. Ritchie

Abstract | Natural killer T (NKT) cells are credited with regulatory roles in immunity against cancers, autoimmune diseases, allergies, and bacterial and viral infections. Studies in mice and observational research in patient groups have suggested that NKT cell-based therapies could be used to prevent or treat these diseases, yet the translation into clinical settings has been disappointing. We support the view that NKT cells have regulatory characteristics that could be exploited in clinical settings, but there are doubts about the natural roles of NKT cells *in vivo* and whether NKT cell defects are fundamental drivers of disease in humans. In this Opinion article, we discuss the uncertainties and opportunities regarding NKT cells in humans, and the potential for NKT cells to be manipulated to prevent or treat disease.

Natural killer T (NKT) cells are a regulatory T cell lineage that has a range of immune activities. These include promoting cell-mediated immunity against tumours, bacteria and viruses; preventing autoimmune diseases through the regulation of self-tolerance; and contributing to allergic-type diseases such as psoriasis and asthma^{1,2}.

Studies of many animal models and patient groups have suggested an association between NKT cell defects and disease. There is a growing interest in using these cells in therapeutic settings as biomarkers for prognostic purposes, as well as developing new treatments that counteract NKT cell defects and/or exploit the unique functions of these cells^{3,4}. We argue that more direct studies of NKT cells in healthy and diseased individuals will better define the natural role of NKT cells and help to determine the diseases in which NKT cell-based therapies will be most effective.

NKT cells: the basics

Defining NKT cells. The term NKT cell has evolved over the past 20 years but it now applies to any CD1d-restricted T cell^{5,6}. Important subclassifications include type 1 NKT cells, which express a semi-invariant T cell receptor (TCR) that is reactive to the glycolipid antigen α -galactosylceramide (α GalCer); and type 2 NKT cells, which collectively express a more diverse TCR repertoire^{5,6}. There is also a 'catch-all' classification of NKT-like cells that applies to MHC-restricted T cells with

characteristics that are associated with type 1 or type 2 NKT cells (for example, recognition of α GalCer). Type 1 NKT cells are by far the most widely studied group and we focus on these cells in this article, with subsequent uses of the term NKT cell referring only to this subset.

How NKT cells are activated. The NKT cell TCR is comprised of an invariant TCR α chain (Va24–Ja18 in humans and the orthologous Va14–Ja18 in mice) and a restricted assortment of TCR β chains (V β 11 in humans; V β 2, V β 7 or V β 8 in mice)⁷. NKT cells from these and other species recognize α GalCer, which is seen as a testament to their evolutionary conservation and probable physiological importance. Synthetic variants of α GalCer are used to study the NKT cell TCR–antigen–CD1d complex and to identify and stimulate NKT cells, and although many foreign lipids are recognized by NKT cells, it is not known whether NKT cells encounter them under non-experimental conditions^{2,7,8}. For example, antigens from *Streptococcus pneumoniae*⁹ and *Borrelia burgdorferi*¹⁰ stimulate NKT cells, but it is not known if this is indicative of an important antimicrobial role for NKT cells, or whether NKT cells normally participate in immune responses against these pathogens. There are other microbial antigens that also stimulate NKT cells and are derived from microorganisms that do not pose a substantial health risk in humans, which could indicate that NKT cells provide effective

protection^{11,12}. In experimental settings, NKT cells also respond to many self lipids and some researchers believe this may be the primary means by which NKT cells are normally activated *in vivo*¹³. Interestingly, interleukin-12 (IL-12) — as well as IL-18, IL-23 and IL-25 — may stimulate NKT cells independently of the TCR^{12,14}. However, the conservation of NKT cell antigen specificity suggests that cytokines are unlikely to be the primary trigger of activation.

The response of activated NKT cells. Once activation occurs, NKT cells can influence immunity by releasing cytokines, including interferon- γ (IFN γ), IL-4, IL-10, IL-13, IL-17, IL-21 and tumour necrosis factor (TNF)¹². The type and quantity of cytokine release differs between mice and humans, and is influenced by antigen signal strength, CD1d-binding kinetics, subcellular localization of lipid-loaded CD1d molecules and antigen targeting to different antigen-presenting cells (APCs)¹². The presence of functionally distinct NKT cell subsets may be important for promoting different outcomes^{15,16}. For example, human CD4⁺ NKT cells mostly release T helper 1 (T_H1)-type cytokines, whereas CD4⁺ NKT cells are associated with a T_H0-type or T_H2-type response^{16–18}.

Association between NKT cells and diseases

NKT cells have been associated with an extraordinary number of different diseases. In this article, we focus on cancer^{19–21} (BOX 1), type 1 diabetes^{22–24} (BOX 2) and asthma^{25–27} (BOX 3), for which the association with NKT cells is the most debated. In each of these diseases, experimental mouse models and/or observational studies in patients suggest that the dysfunction or pathogenic activity of NKT cells could be important in the disease process. Each disease has been suggested as a potential candidate for NKT cell-based therapies, but the importance of NKT cells in these disease settings is far from certain and we argue that more direct studies aimed at defining the role of NKT cells in patients will help to identify settings in which NKT cell-based therapies will be effective.

NKT cells and cancer. Although NKT cell-deficient mice are generally healthy and long lived, they have a higher incidence of tumour development and more rapid tumour progression in spontaneous and carcinogen-induced models²⁰. In some instances, adoptive transfer or stimulation of NKT cells in mice can be protective^{20,28–31}. NKT cell defects have been reported in patients with solid or haematological cancers^{20,32–34},

including cancers in which NKT cell frequency correlated positively with prognosis and inversely with tumour load³⁵. There are interesting associative studies suggesting that NKT cells promote graft-versus-leukaemia effects and suppress graft-versus-host disease after haematopoietic stem cell transplantation^{36,37}, and there are instances in which the administration of α GalCer-loaded APCs and activated NKT cells has led to moderate clinical benefits^{38,39}. Unfortunately, the rarity of human NKT cells and low patient numbers have limited the scope of many studies and there is a poor understanding of whether NKT cell function or subset distribution is abnormal in these patients^{3,20}. Most studies have analysed the overall frequency of NKT cells in the blood, and that of CD4⁺ and CD4⁻ NKT cell subsets, but it would be interesting to know more about how these cells are behaving and, in particular, whether they are responding to the tumour cells. For example, analysing the spontaneous and inducible cytokine responses of NKT cell subsets would be revealing because CD4⁻ NKT cells normally have a pro-inflammatory T_H1-type cytokine profile, whereas CD4⁺ NKT cells are seen as more tolerogenic^{15,16,18}. It is easy to see the potential significance of abnormal cytokine profiles in a disease setting. Without this depth of analysis, debate about the antitumour activity of human NKT cells has been over-reliant on observational studies of NKT cell frequency in patients, or on extrapolating findings from *in vitro* assays or mouse models, and there is very little information about where, how and if NKT cells are naturally responding to cancer in these patients. A recurring problem is that most human studies have only analysed NKT cells from the blood, so little is known about the NKT cells that are present elsewhere in the body (such as within the tumour microenvironment) and whether these cells are responding at all. A better understanding of how NKT cells naturally respond to tumours in humans is needed. A more detailed overview of the association between NKT cells and cancer is provided in BOX 1 (for reviews, see REFS 19–21).

NKT cells and type 1 diabetes. There is a long-standing debate about whether NKT cell defects contribute to type 1 diabetes^{3,24}. The evidence in mice is quite strong, as diabetes-prone non-obese diabetic (NOD) mice are NKT cell deficient, their residual NKT cells are functionally defective and protection can be conferred by stimulating the residual NKT cells with α GalCer or by adoptive transfer of thymus-derived NKT cells⁴⁰.

Box 1 | NKT cells and cancer

Observations relating to the association between natural killer T (NKT) cells and cancer are listed in approximate chronological order.

- The prototypic NKT cell antigen, α -galactosylceramide (α GalCer), was identified as a potential anticancer agent during a screen of marine sponge-derived glycolipids⁷⁰.
- α GalCer-reactive NKT cells were shown to be important for effective antitumour immunity in several cancer models, including conflicting data in methylcholanthrene-induced fibrosarcomas^{71,72}, as well as studies in tumour-prone p53-hemizygous mice³¹, B16 melanoma¹⁵ and transgenic adenocarcinoma of mouse prostate cancer⁷³.
- Adoptive transfer experiments in mice have suggested that CD4⁻ NKT cells are the main antitumour NKT cell subset¹⁵.
- NKT cells were shown to recognize and lyse some CD1d⁺ tumour cells and those that are loaded with α GalCer^{74–76}.
- Many solid tumours do not express CD1d. CD1d expression by tumour cells was deemed unnecessary in some NKT cell-dependent models, which suggests third-party presentation of cancer-derived antigens or non-contact-dependent mechanisms of tumour control⁷⁶.
- Interferon- γ (IFN γ) release by NKT cells was shown to be important in several models, leading to: the stimulation of NK cells⁷¹; the licensing of dendritic cells (DCs) to release interleukin-12 (IL-12) and promote development of antitumour cytotoxic T lymphocytes; and the inhibition of tumour angiogenesis through effects on CD1d⁺ tumour-infiltrating macrophages⁷⁷.
- α GalCer has been used in clinical trials to promote NKT cell proliferation and cytokine release *in vivo* in patients with cancer^{20,78}.
- The peripheral blood of patients with solid cancers was shown to have a deficiency of NKT cells and there were settings in which NKT cell frequency correlated inversely with tumour load and positively with prognosis³².
- In patients with advanced head and neck cancer, NKT cell frequency was found to be unaffected but NKT cell proliferation was defective⁷⁹.
- In some cases, relative NKT cell deficiencies in tumours and the surrounding tissues were found^{80,81}, although increased NKT cell frequency was also found in some tumours^{82,83}. In one study, a correlation was observed between a higher NKT cell frequency and improved prognosis⁸².
- Reduced NKT cell frequency and function was found in patients with haematological cancers, including multiple myeloma (MM)⁸⁴ and myelodysplastic syndromes^{85–87}. Recent studies contrast with these findings and question whether NKT cells are defective in these patient groups^{88,89}.
- Clinical trials have shown that α GalCer is safe in humans but it is largely ineffective when delivered intravenously and may lead to NKT cell anergy^{52,90}.
- Patients who are injected with DCs that have been pulsed with α GalCer showed transient increases in NKT cell frequency and higher serum concentrations of IFN γ and IL-12 (REF. 58).
- Compared with the administration of α GalCer, patients receiving tumour cells or antigen-presenting cells that had been pulsed with α GalCer showed improved antitumour responses^{21,38,39}. Objective clinical benefits were absent or modest, but this remains an active area of clinical investigation.
- Co-injecting α GalCer and tumour antigens was shown to be a potential alternative approach^{55,91}.
- NKT cells were shown to promote graft-versus-leukaemia effects and suppress graft-versus-host disease in patients after haematopoietic stem cell transplantation^{36,37}.
- Polarizing the NKT cell response has been partly successful in mice⁹² and humans⁹³, although polarization is slow to develop and is reversible. Ongoing doses of glycolipid are required for maximal effect.
- The immunomodulatory drug lenalidomide was shown to selectively increase NKT cell responses to α GalCer *in vitro* and in patients with MM^{94–96}. By contrast, our studies in patients with MM or myelodysplastic syndromes did not show any lenalidomide-induced changes in NKT cell frequency or function^{88,89}.
- The immune cell subsets found in peripheral blood may not accurately reflect systemic cancer immunity⁶⁴.
- NKT cell frequency was found to be higher in lung tumours than in the blood⁹⁷, and increased NKT cell frequency in the microenvironment of colorectal cancers may be a positive prognostic indicator⁸². However, there is uncertainty about the stringency of the analysis that has been carried out in some studies.

Box 2 | **NKT cells and type 1 diabetes**

Observations relating to the association between natural killer T (NKT) cells and type 1 diabetes are listed in approximate chronological order.

- NKT cells may have a natural role in self-tolerance and preventing autoimmune disease⁴¹, as there is an association between NKT cell defects and autoimmune diseases such as type 1 diabetes²³.
- NKT cell frequency was shown to be reduced and the remaining NKT cells dysfunctional in non-obese diabetic (NOD) mice^{67,98,99}.
- Several studies showed that restoring the NKT cell pool in NOD mice — through adoptive transfer^{98,100,101}, transgenesis^{67,102}, congenesis⁴⁰ or by stimulation with α -galactosylceramide (α GalCer)^{103,104} — significantly reduced diabetes incidence.
- The mechanisms of NKT cell-mediated protection in NOD mice are unclear. Cytokine release by NKT cells was suggested to be important in some²³, but not all¹⁰⁵, studies.
- Other suggested mechanisms of protection include NKT cell-mediated skewing of the cytokine profile of autoreactive T cells from a T helper 1 (T_H1) to a T_H2 type¹⁰⁶, promoting the differentiation and mobilization of tolerogenic dendritic cells (DCs)²³, enhancing the differentiation and impact of forkhead box P3 (FOXP3)-expressing regulatory T (T_{Reg}) cells^{107,108} or inhibiting interleukin-17 (IL-17) release through the production of IL-4 (REF. 23). However, IL-17-producing NKT cells could potentially exacerbate disease¹²⁰.
- CD1d deficiency in NOD mice was shown to increase the incidence and progression of diabetes¹⁰⁹, however another study found that NKT cell-mediated protection was independent of CD1d¹⁰⁵.
- NKT cell defects are found in patients with type 1 diabetes and in individuals who are at high risk of developing the disease¹¹⁰.
- More recent studies have reported that NKT cell numbers are normal or increased in patients with type 1 diabetes or those in risk groups^{111–113}.
- Reduced IL-4 production by NKT cells from pancreatic lymph nodes was observed in patients with type 1 diabetes¹¹⁴.
- Potential therapeutic uses of α GalCer analogues include promoting T_H2 -type cytokine release, boosting NKT cell numbers, and inducing tolerogenic DCs and T_{Reg} cells²³.

Cytokine release by NKT cells is probably important for this protection, but the mechanisms by which endogenous NKT cells provide protection *in vivo* are not understood.

In humans, at least ten studies have failed to reach a consensus about the importance of NKT cells in type 1 diabetes^{3,23,41,42}. NKT cell deficiencies, subset disturbances and abnormal cytokine production have all been reported in patients with type 1 diabetes or those who are at high risk of developing the disease, but other studies of a seemingly similar design have found different defects or no defects at all (BOX 2). The conflicting results cannot easily be explained and thus NKT cells cannot be confidently used as a biomarker to stratify patients or as a target of new immunotherapies for type 1 diabetes^{22,41}. A more detailed overview of the association between NKT cells and type 1 diabetes is provided in BOX 2 (for reviews, see REFS 22–24).

NKT cells and asthma. Unlike cancer and type 1 diabetes, in which a loss of NKT cell function (through deficiency or dysfunction) reportedly predisposes to disease, it is the unwelcome activities of NKT cells that seem to contribute to asthma. As with cancer and type 1 diabetes, evidence from mouse models of asthma led to investigations of NKT cells in human patients. In mice, NKT cells

have been implicated in several models of asthma²⁵. These include virus- and allergy-induced models in which cytokine release from NKT cells exacerbates asthma directly, or indirectly via lung monocytes and macrophages^{26,123}. It was then reported that there were vastly increased numbers of NKT cells in the lungs of humans with asthma⁴³. This brought NKT cells to the forefront of the asthma field but, despite some supporting evidence that NKT cells contribute to asthma^{44,46}, several later studies questioned the methodology of the original (and some subsequent) studies and demonstrated that cells from bronchoalveolar lavage fluid and lung tissues required very stringent flow cytometry analysis to exclude inherently high background staining and autofluorescence^{45–47}. The role of NKT cells in asthma remains uncertain, although there seems to be a correlation between increased NKT cell frequency and the most severe forms of allergic asthma²⁶. A more detailed overview of the association between NKT cells and asthma is provided in BOX 3 (for reviews, see REFS 25–27).

Potential clinical applications of NKT cells

The reported association between NKT cells and a range of diseases implies that NKT cells could be exploited in the prevention, diagnosis and/or treatment of these diseases.

For example, NKT cell characteristics could be used as biomarkers to stratify individuals with different prognostic outcomes. Alternatively, more direct clinical benefits could be achieved by stimulating (or adoptively transferring) NKT cells to correct defects within the NKT cell pool, or to harness their regulatory or agonistic effects. In this section, we discuss these prospects in the context of disease settings in which NKT cells are thought to have a protective role and in which NKT cell defects have been identified in the blood of patients with, for example, cancer and autoimmune disease.

NKT cells as biomarkers. For diseases in which patients reportedly have deficiencies in circulating NKT cells or their subsets^{19,41,48}, one might reasonably assume that NKT cell frequency could be used to stratify patients into groups that are more or less likely to progress and/or respond to treatment. However, the high variability and Poisson distribution of NKT cell frequency in the blood of healthy individuals makes it exceedingly difficult to reliably categorize individuals as deficient^{49,50}. In addition, most studies have only examined patients on a single occasion after disease onset, so correlations between NKT cell characteristics (for example, altered subset ratios and function) and disease progression are poorly defined for most diseases, and it mostly remains uncertain whether NKT cell defects are a cause or consequence of the disease process³.

Counteracting NKT cell defects that contribute to disease.

There is great interest in using α GalCer or related lipids to restore or improve NKT cell function in patients with diseases that are associated with NKT cell defects. Clinical trials have established that α GalCer is mostly safe in humans, but there are varied reports of how effectively it can stimulate NKT cells and increase their frequency and cytokine production^{20,51}. The administration of soluble α GalCer causes NKT cell stimulation but may lead to NKT cell anergy^{52–54}. The approach has mostly been superseded by the administration of α GalCer-loaded APCs but this typically generates only a moderate (approximately threefold) and transient spike in NKT cell frequency in the blood before numbers return to baseline, with many patients showing little response³⁸. The fact that these patients may have underlying NKT cell defects complicates the administration and evaluation of NKT cell-based treatments, but another issue is whether NKT cell defects actually

contribute to disease and if counteracting this defect will benefit the patient. This causal relationship is suspected but not proven in several human diseases and more studies are needed. Of course, it is still feasible that stimulating NKT cells will be beneficial, even if NKT cells do not normally affect the immune responses that are associated with the disease, but further advances will benefit from a greater understanding of the roles of NKT cells in disease.

NKT cells as adjuvants. The potent cytokine production of NKT cells after stimulation warrants investigation of whether these cells can be harnessed as a cellular adjuvant to improve vaccines and adoptive cell therapies, or to modulate immune responses for therapeutic benefit^{12,55,56}. α GalCer has been safely trialled in humans and the administration of α GalCer-pulsed dendritic cells has resulted in measurable NKT cell activation, although demonstrable clinical benefits have been modest^{2,20,39}. Future trials will probably involve the administration of tumour cells that have been pulsed with α GalCer or the adoptive transfer of NKT cells that have been expanded or activated *in vitro*, and various combination therapy approaches that involve NKT cells, α GalCer and conventional treatments^{57,58}.

The caveats of using NKT cells as adjuvants include the highly variable NKT cell frequency in humans and the use of patient groups that reportedly have NKT cell defects, which may reduce the effectiveness of targeting NKT cells in these individuals. Some studies have reported that only patients with high NKT cell frequency were likely to benefit from NKT cell-based treatments, which is problematic if a deficit of NKT cells is associated with the disease³⁹. The ability of α GalCer to act as a global NKT cell agonist is also problematic because the stimulation of all NKT cells — including subsets with counter-regulating cytokine profiles — may result in unexpected, antagonistic or unwanted results. However, approaches that use different lipid agonists to selectively promote particular cytokine profiles hold promise for more refined therapeutic applications^{7,12,59}.

The natural role of NKT cells

Most translational studies involving NKT cells have focused on patient groups with NKT cell defects or those in which NKT cells are thought to have a natural role in preventing the disease. We have summarized the associations between NKT cell defects and human diseases, and it is important to consider whether the natural involvement of

Box 3 | NKT cells and asthma

Observations relating to the association between natural killer T (NKT) cells and asthma are listed in approximate chronological order.

- Airway hypersensitivity in mouse models of allergic asthma was shown to depend on NKT cells that produce interleukin-4 (IL-4) and IL-13 (REF. 115), which are cytokines that were previously linked to asthma¹¹⁶ (for a review, see REF. 117).
- Airway hypersensitivity was shown to occur normally in NKT cell-deficient mice¹¹⁸. However, contrasting results have been reported, possibly owing to differences in the modelling of asthma and the nature of the NKT cell deficiency in the respective studies^{115,118}.
- A mouse model of airway hypersensitivity was shown to depend on NKT cells and IL-17 (REF. 119).
- Bronchoalveolar lavage fluid samples from patients with asthma were shown to have very high numbers of NKT cells (more than 50% of the total CD4⁺ T cells)⁴³.
- NKT cells are thought to migrate to the lungs of patients with asthma, as they are essentially absent from the lungs of healthy donors and those of patients with an unrelated respiratory condition⁴³. This suggested NKT cells were central to the release of T helper 2 (T_H2)-type cytokines in the airways, rather than conventional MHC class II-restricted T cells, as was previously suspected¹²¹.
- In patients with asthma, CD4⁺ NKT cells from the airways have been shown to produce IL-4 and IL-13 (but little interferon- γ), but those in the blood were not biased towards the T_H2-type cytokines, were not activated and were present at normal frequencies⁴³.
- Disagreements emerged about the stringency of flow cytometry analysis, patient history and methodology that were used to show increased NKT cell numbers in patients with asthma⁴³, and it was suggested that the frequency of these cells was overestimated because doublets and autofluorescent, non-viable cells were not fully excluded⁴⁷. The debate is ongoing^{45–47}.
- It seems most likely that the NKT cell frequency in the lungs was overestimated, but that NKT cells are sometimes present in the lungs of patients with asthma (particularly in those with severe asthma or following allergic challenge) at a higher frequency than is normally seen in healthy individuals²⁶.
- Activation of NKT cells in the lungs of mice¹²² and monkeys⁴⁴ was shown to worsen the symptoms of allergic asthma, but it is not clear whether NKT cells become activated in human patients with asthma or whether activation would have the same pathogenic effects as it does in mice. In addition, it is not clear whether NKT cells in the lungs of patients with asthma naturally produce cytokines and what effects these cytokines might have.

human NKT cells in preventing disease has been overstated. The point is not to argue against the potential benefits of NKT cell-based therapies, but rather to ask whether the patient groups that will benefit most from therapeutic manipulation of NKT cell cytokine production are necessarily those in which NKT cells have a natural role in preventing that disease. In this section, we consider whether the characteristics of human NKT cells support or challenge the concept of NKT cells as natural, spontaneous responders that prevent diseases in humans, and as potential clinical tools for the prevention, diagnosis and/or treatment of disease.

NKT cell frequency. The average frequency of NKT cells in humans is ~0.01% of total peripheral blood mononuclear cells, but this varies ~100-fold between healthy individuals without any obvious impact on health or immunity. This calls into question whether there is a threshold NKT cell frequency below which a 'deficiency' becomes clinically relevant, and whether there is value in monitoring (and treating) NKT cell deficiency as a biomarker of disease susceptibility. The high variability of NKT cell frequency in humans

also means that large numbers of replicates are needed to identify deficiencies in patient groups, and that NKT cell-deficient individuals cannot be easily distinguished unless the definition is so extreme that it renders the classification worthless for identifying high-risk individuals. A related issue is that the low and very variable frequency of NKT cells could make it difficult to develop standard therapies that generate predictable NKT cell responses without pre-screening each patient for NKT cell characteristics such as subset frequency and function^{39,42}.

Applicability of NKT cell studies in mice to humans.

Aside from well-documented arguments about the suitability of inbred mice as a model of human immunity⁶⁰, NKT cells might simply be less important in humans than in mice. Diabetes-prone NOD mice are deficient in NKT cells, which renders them defective for NKT cell-mediated tolerance^{23,24}. By contrast, the NKT cell frequency in most healthy humans is often more than fivefold lower than that of NOD mice and tenfold lower than that of wild-type mice^{3,23,64}. This raises questions about whether NKT cells have the same influence on systemic immunity in humans,

particularly as NKT cell-deficient mice on wild-type backgrounds are healthy and long lived. Some investigators have speculated that NKT cells could be functionally redundant in humans and that their functions are possibly carried out by lineages such as mucosa-associated invariant T (MAIT) cells⁶¹ — which have similar functional characteristics to NKT cells and are abundant in humans but almost undetectable in mice^{61,62} — and CD1a-, CD1b- and CD1c-restricted T cell subsets². A more specific issue for the NKT cell field is that the most commonly studied NKT cell-deficient mice (*TraJ18*^{-/-} mice, which lack the gene encoding the J α 18 TCR α -chain) have an unexpectedly restricted TCR diversity compared with wild-type mice⁶³. This seems to be the result of introducing a neomycin resistance gene when generating this strain. However, regardless of cause, this raises important questions about whether the increased susceptibility of NKT cell-deficient mice to cancers and other diseases should still be attributed to a lack of NKT cells, or whether the reduced diversity of the conventional T cell pool is a contributing factor. Unfortunately, the findings are not easily verified using CD1d-deficient mice (which have normal TCR diversity in their conventional T cell populations) because these mice lack type 2 NKT cells, which are now emerging as an important subset⁷.

Where are activated NKT cells in non-experimental settings? In mice, NKT cells are present at a low frequency (accounting for ~1% of T cells) in most tissues (such as the blood, spleen and thymus) and are comparatively over-represented (accounting for 30–50% of T cells) in the liver¹²⁴. In humans, NKT cells have a similar distribution pattern but the frequencies are approximately tenfold lower. The strategic purpose of this distribution is not understood and, at face value, it seems inconsistent with the systemic and tissue-specific diseases that are associated with NKT cells³. Given the crucial roles attributed to these cells in preventing cancer, autoimmunity and microbial infections, it seems remarkable that there are few examples of spontaneous NKT cell responses occurring without prior administration of α GalCer or other experimental agonists.

If NKT cells regulate immunity through cytokine release, why don't we readily observe NKT cells releasing cytokines in patients with cancer, type 1 diabetes or other conditions in which these cells are thought to be important? An important consideration is that human studies often only use blood samples as a source of NKT cells and studies

in mice show that the blood offers a poor reflection of systemic NKT cell frequency and function⁶⁴. This leaves open the possibility that NKT cells are activated in other tissues, but it should be feasible to test this. To date, there is little evidence of NKT cell migration into tumours or of their activation and proliferation in these patients.

Of course, a failure to observe activation is not evidence of inactivity. For instance, there have only been rare examples of spontaneous (that is, not induced by administering α GalCer or other lipid agonists) NKT cell activation or inflammation-associated NKT cell migration in mice⁶⁵, despite convincing evidence that NKT cells are important. Activated NKT cells have also been identified in the peripheral blood of patients with sickle cell disease⁶⁶ and in T cell-reactive granulomas⁶⁵. However, the lack of evidence that human NKT cells respond spontaneously in diseases such as cancer seems inconsistent with them having an important natural role in these settings. More studies are needed to address how, why and where NKT cells are naturally activated in non-experimental settings that do not involve the administration of lipid agonists.

Subset studies. Mouse-based studies have mostly used the thymus, spleen or liver as a source of NKT cells for functional assays, yet human studies rely almost exclusively on cells from the blood. There are dangers in extrapolating information about one tissue to others, as NKT cells from different tissues — and even those from the same tissue in mice and humans — often have very different characteristics. For example, most NKT cells in the mouse thymus are functionally mature, but NKT cells in the human thymus are 100-fold less abundant, and phenotypically and functionally immature¹²⁴. We need to be cautious about over-interpreting the significance of studies that mainly rely on data from mouse thymic NKT cells — including functional characterization studies of NKT cells and demonstrations of NKT cell-mediated protection in NOD mice — because there may not be an equivalent NKT cell subset in humans. The difficulties are further illustrated in subset characterization, as adoptive transfer experiments in mice have shown that CD4⁻ NKT cells are more effective than CD4⁺ NKT cells in mediating tumour rejection¹⁵. Confusingly, whereas human CD4⁻ and CD4⁺ NKT cells from the blood have cytokine differences that align well with their proposed pro-inflammatory and tolerogenic roles, respectively, this is not observed in mice, although the cytokine profiles of mouse NKT cell subsets do differ^{68,69}. It is difficult

to be definitive about the antitumour role of CD4⁻ NKT cells, given that efficacy has only been demonstrated in mice and that a T_H1 versus T_H0 cytokine differential is only observed in humans. Further investigation is required to fill such gaps in our understanding and we will need to be cautious about over-interpreting the significance of mouse studies that characterize the functions of NKT cells in the absence of supporting evidence in humans.

Functional heterogeneity. A long-standing question is how NKT cells can promote immunity against cancers, virus and bacteria, but also suppress autoimmunity. Functionally distinct subsets could mediate different activities or different forms of stimulation could drive diverse functional outcomes, but it is not understood how this would apply in natural settings for preventing cancer and autoimmunity. The full extent of functional heterogeneity in the NKT cell pool is yet to be determined, but for the existence of subsets to explain the functional versatility of NKT cells *in vivo*, different subsets must be selectively activated. How is this possible when NKT cell subsets have a similar specificity for antigen? Analogues of α GalCer can induce different cytokine profiles but polarization seems to mostly rely on the properties of the antigen and the APCs⁷, which is difficult to reconcile with NKT cell subsets correctly determining whether to promote or suppress particular immune responses. Selective migration is also a possibility, but NKT cell subsets do not seem to migrate independently and we are not aware of examples in which antigens (or cytokines) differentially activate NKT cell subsets in disease settings. There is little direct evidence that NKT cells become activated in diseases such as cancer or type 1 diabetes, and it is still not known whether they act systemically or have local effects within tissues. Discoveries in these areas may provide clues about the functional versatility of NKT cells but it is frustrating that these issues remain unresolved.

Concluding remarks

"Absence of evidence is not evidence of absence" Carl Sagan

There is convincing evidence that NKT cells are important in mice but it is not known where these cells are activated, what they respond to or where they mediate their function when participating in spontaneous responses against cancer or providing protection against autoimmunity. Although we

argue that it is important to consider whether NKT cells have a lesser role in humans than in mice, it is also important to acknowledge that direct evidence for NKT cell involvement in human diseases may be uncovered in the future. Thus, a crucial next phase of research is to search for direct evidence of NKT cell activity in human disease settings. For example, studying activated NKT cells in patients with cancer would substantially advance our understanding of the NKT cell contribution to antitumour responses and help to predict whether NKT cell-based therapies would be beneficial in this context. However, we must also be willing to accept the null hypothesis — that is, NKT cells may not be as important in humans as some studies have suggested. Striking the right balance between these boundaries provides the greatest chance of developing NKT cell-based approaches that improve our ability to diagnose, prevent and treat diseases.

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Competing interests statement

The authors declare no competing interests.