REVIEWS

Targeting natural killer cells and natural killer T cells in cancer

Eric Vivier[1,2,3,4], Sophie Ugolini[1,2,3,5], Didier Blaise[5,6,7], Christian Chabannon[5,6,7] and Laurent Brossay[8]

Abstract | Natural killer (NK) cells and natural killer T (NKT) cells are subsets of lymphocytes that share some phenotypical and functional similarities. Both cell types can rapidly respond to the presence of tumour cells and participate in antitumour immune responses. This has prompted interest in the development of innovative cancer therapies that are based on the manipulation of NK and NKT cells. Recent studies have highlighted how the immune reactivity of NK and NKT cells is shaped by the environment in which they develop. The rational use of these cells in cancer immunotherapies awaits a better understanding of their effector functions, migratory patterns and survival properties in humans.

[1]*Centre d'Immunologie de Marseille-Luminy, Université d'Aix-Marseille, Campus de Luminy Case 906, 13288 Marseille, France.*
[2]*INSERM UMR 1104, Marseille, France.*
[3]*CNRS, UMR 7280, Marseille, France.*
[4]*Assistance Publique – Hôpitaux de Marseille, Hôpital de la Conception, 13385 Marseille, France.*
[5]*Institut Paoli-Calmettes, Centre de Lutte Contre le Cancer, Marseille, France.*
[6]*Université d'Aix-Marseille, Marseille, France.*
[7]*INSERM CBT-510, Centre d'Investigations Cliniques en Biothérapie, Marseille, France.*
[8]*Department of Molecular Microbiology and Immunology, Division of Biology and Medicine, Brown University, Providence, Rhode Island, USA.*
Correspondence to E.V. and L.B.
e-mails:
vivier@ciml.univ-mrs.fr;
laurent_brossay@brown.edu
doi:10.1038/nri3174

The immune system is classically divided into innate and adaptive branches. The adaptive immune system can be defined by the presence of cells (B cells and T cells in higher vertebrates) that can respond to many diverse environmental antigens. This is achieved through the clonal expression of a colossal repertoire of B cell receptors (BCRs) and T cell receptors (TCRs) with distinct antigen specificities, the diversity of which results from somatic DNA rearrangements. By contrast, the recognition of various assaults by cells of the innate immune system has been shown to depend on germline-encoded receptors. A recent paradigm shift in our understanding of immunity in mammals has resulted from the discovery of the recognition receptors used by the innate immune system, including the Toll-like receptors (TLRs)[1–3].

Besides conventional B and T cells, several populations of innate lymphoid cells (ILCs) were recently identified[4]. ILC populations include various cells of the innate immune system, such as lymphoid tissue-inducer (LTi) cells, but also cells that produce interleukin-5 (IL-5), IL-13, IL-17 and/or IL-22 and that help to initiate immune responses to pathogens. Natural killer cells (NK cells) are now recognized as a subset of cytotoxic ILCs that express the transcription factor E4BP4 (E4 promoter-binding protein 4; also known as NFIL3). NK cells secrete cytokines, such as interferon-γ (IFNγ), that participate in the shaping of the adaptive immune response[5]. An important feature of NK cells is their capacity to distinguish stressed cells (such as tumour cells, infected cells and cells that have undergone physical or chemical injuries) from healthy cells. NK cells were initially identified through their ability to kill tumour cells (hence their name)[6–8]. Since then, the antitumour effect of NK cells has been documented in many models and instances. *In vitro*, mouse and human NK cells can kill a broad range of tumour cells of haematopoietic and non-haematopoietic origin. *In vivo*, mouse NK cells can eliminate many transplantable and spontaneous tumours[9,10]. Selective NK cell deficiencies are extremely rare[11], thus preventing the monitoring of cancer incidence in these patients, but also possibly testifying to the physiological importance of NK cells. Nevertheless, an epidemiological study has linked low peripheral blood NK cell activity with increased cancer risk[12]. In addition, NK cell infiltration into tumour tissue is associated with better disease prognosis in non-small cell lung carcinomas[13,14], clear cell renal cell carcinomas[15] and colorectal cancer[16]. Observations in patients with advanced gastrointestinal stromal tumours treated with imatinib mesylate support the hypothesis that NK cells exert antitumour effects not only through direct cytolytic activity, but also indirectly through their ability to produce cytokines such as IFNγ[17].

Natural killer T (NKT) cells have been classified into four different groups[18]. Only type I and type II NKT cells are CD1d-restricted; they respond to CD1d-expressing cells and are absent in CD1d-deficient mice[18] (BOX 1). The most-studied group — known as type I NKT cells or invariant NKT cells (iNKT cells) — is well conserved in mammals[18]. iNKT cells develop in the thymus and arise from the same common lymphoid precursor pool as conventional T cells[19,20]. After αβ T cell lineage commitment and the generation of double-positive thymocytes, the iNKT cell and conventional T cell selection pathways diverge[21,22]. iNKT cell precursors are selected following

Box 1 | **CD1d-restricted T cells and the activation of iNKT cells**

Cells that are activated in response to antigens presented by the MHC class I-like molecule CD1d are highly conserved in mammals and have been classified as type I and type II natural killer T (NKT) cells[18]. Type I NKT cells are also known as invariant NKT (iNKT) cells and express a semi-invariant T cell receptor (TCR), whereas type II NKT cells express a more diverse TCR repertoire. iNKT cells have been characterized using CD1d tetramers loaded with α-galactosylceramide (αGalCer) in both mice and humans, and also by using the 6B11 monoclonal antibody in humans[218–220]. By contrast, the functions of type II NKT cells are unclear and have only been indirectly examined by comparing the phenotypes and immune responses of CD1d-deficient and Jα18-deficient animals, which lack iNKT and type II NKT cells and only iNKT cells, respectively.

iNKT cell frequencies are lower in humans than in mice, but the frequencies of type II NKT cells in both species are unknown owing to a lack of reliable tools to identify these cells. Although all iNKT cells can be identified by staining with CD1d tetramers, recent data indicate that there are several distinct subsets of iNKT cells with different functions and phenotypes. Notably, although most iNKT cells express the Vα14–Jα18 TCR α-chain, an αGalCer-reactive NKT cell subset that expresses a Vα10–Jα50 TCR α-chain was recently identified[221].

iNKT cells are unique because they have the ability to respond both as innate cells, with minimal TCR involvement, and as memory-like cells through the engagement of their semi-invariant TCR. TCR-dependent activation of iNKT cells has been extensively studied using the strong agonist αGalCer, which is presented on CD1d. In this case, the iNKT cell response is CD1d dependent, and pro-inflammatory cytokines are dispensable. Interestingly, however, iNKT cell-mediated protection against pathogens that express antigens recognized by the invariant TCR of iNKT cells predominantly depends on pro-inflammatory cytokines, such as interleukin-12 (IL-12)[47].

The indirect activation of iNKT cells has recently been characterized during viral infection and is induced in the presence of large amounts of pro-inflammatory cytokines, such as IL-12, IL-18 and interferon-α (IFNα)[43–45]. In this setting, iNKT cells functionally resemble classical natural killer (NK) cells and produce mostly T helper 1 (T_H1)-type cytokines. iNKT cells can also be activated by combined exposure to pro-inflammatory cytokines and CD1d-dependent TCR stimulation. This has been reported to occur during infection with certain bacteria (such as *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Staphylococcus aureus* and *Mycobacterium tuberculosis*) that do not themselves express glycolipid agonists for the invariant TCR. Instead, it is thought that iNKT cells become activated in this setting in response to endogenous glycosphingolipids (such as β-D-glucopyranosylceramide[222]) that are presented by pathogen-activated dendritic cells[223–226].

Toll-like receptors
(TLRs). A family of evolutionarily conserved pattern-recognition receptors. These molecules are located intracellularly and at the cell surface of macrophages, dendritic cells, B cells and intestinal epithelial cells. Their natural ligands are molecules that are found in bacteria, viruses and fungi.

Innate lymphoid cells
(ILCs). A group of cells of lymphoid origin that includes NK cells, LTi cells and other non-T, non-B cells that produce distinct cytokines such as IL-5, IL-13 or IL-17.

Natural killer cells
(NK cells). Non-T, non-B lymphocytes that can mediate natural killing of prototypical NK cell-sensitive targets (such as K562 cells in humans and YAC1 cells in mice) and/or produce IFNγ. In humans, NK cells typically have a $NKp46^+CD56^+CD3^-$ phenotype, and they are $NKp46^+NK1.1^+CD3^-$ in the C57BL/6 mouse strain and $NKp46^+CD3^-$ in all mouse strains.

Imatinib mesylate
A first of its class tyrosine kinase inhibitor with clinical activity against chronic myeloid leukaemia associated with the t(9;22) reciprocal translocation. The introduction of imatinib mesylate into clinical practice at the end of the twentieth century induced a rapid shift in medical practices, such that allogeneic haematopoietic stem cell transplantation was abandoned as the standard treatment for this type of cancer.

CD1d
An MHC-like molecule that associates with β2-microglobulin and presents lipids.

Invariant NKT cells
(iNKT cells). A subset of T cells that possess a semi-invariant TCR. In both mice and humans, iNKT cells recognize ligands presented by CD1d.

rearrangement of the TCR α-chain gene and express a semi-invariant TCR (Vα14–Jα18 in mice; Vα24–Jα18 in humans)[21,23–26]. In contrast to conventional T cells, which are selected by antagonist or partial ligands, it is thought that iNKT cells are selected by agonist ligands[27,28]. iNKT cells respond rapidly to a variety of glycolipids presented on CD1d[29] (BOX 1), and strong iNKT cell agonists, such as α-galactosylceramide (αGalCer), stimulate the immediate release of high levels of cytokines[30–33]. Similarly to NK cells, iNKT cells have been shown to have a role in tumour immunosurveillance[34–37], and the potential of these cells in tumour therapy is beginning to be uncovered.

Although their developmental programmes are controlled by different transcription factors (PLZF (promyelocytic leukaemia zinc finger protein; also known as ZBTB16) and E4BP4, respectively)[38–41], iNKT and NK cells have phenotypical and functional similarities. For example, NK cells use LY49H (also known as KLRA8) — a receptor that could be considered to be an invariant TCR-like receptor — to recognize the mouse cytomegalovirus (MCMV) protein m157, and they functionally behave as innate T cells[42]. Conversely, during certain viral infections, iNKT cells can be activated by pro-inflammatory cytokines with minimal TCR involvement and functionally behave as NK cells[43–45]. In addition, both NK and iNKT cells are poised to secrete cytokines[46] and depend on IL-15 and T-bet for their maturation and homeostasis[47–49]. However, unlike NK cell cytotoxicity, which is mediated mostly through perforin- and granzyme-mediated mechanisms, iNKT cell cytotoxicity is mostly restricted to the CD95–CD178 pathway[50].

Here, we discuss recent research on the role of NK and NKT cells in the control of tumours, with a special emphasis on how the molecular dissection of their mode of recognition of tumour cells is leading to the development of innovative cancer therapies. The heterogeneity of NK and NKT cells, their roles in infections and autoimmunity, and their regulatory function in shaping adaptive immunity will not be discussed here because these topics have been extensively covered elsewhere.

How do NK cells recognize tumour cells?

NK cells express an array of receptors that enable them to detect their cellular targets while sparing normal cells. These include inhibitory, activating, adhesion and cytokine receptors. The integration of these signals determines whether or not an NK cell becomes activated (FIG. 1). How this leads to a commensurate NK cell response remains to be determined.

Missing self and NK cell education. NK cells express inhibitory receptors specific for MHC class I molecules (BOX 2). These receptors and their ligands (H-2 molecules in mice and HLA molecules in humans) are highly polymorphic molecules encoded by multigenic, multiallelic families of genes that are inherited independently[51,52]. Thus, NK cells have to discriminate self in a context in which self molecules differ from individual to individual. Similarly to T cells, NK cells are educated to enable self versus altered-self discrimination, but the molecular strategies involved in this education are different. T cell education involves TCR activation,

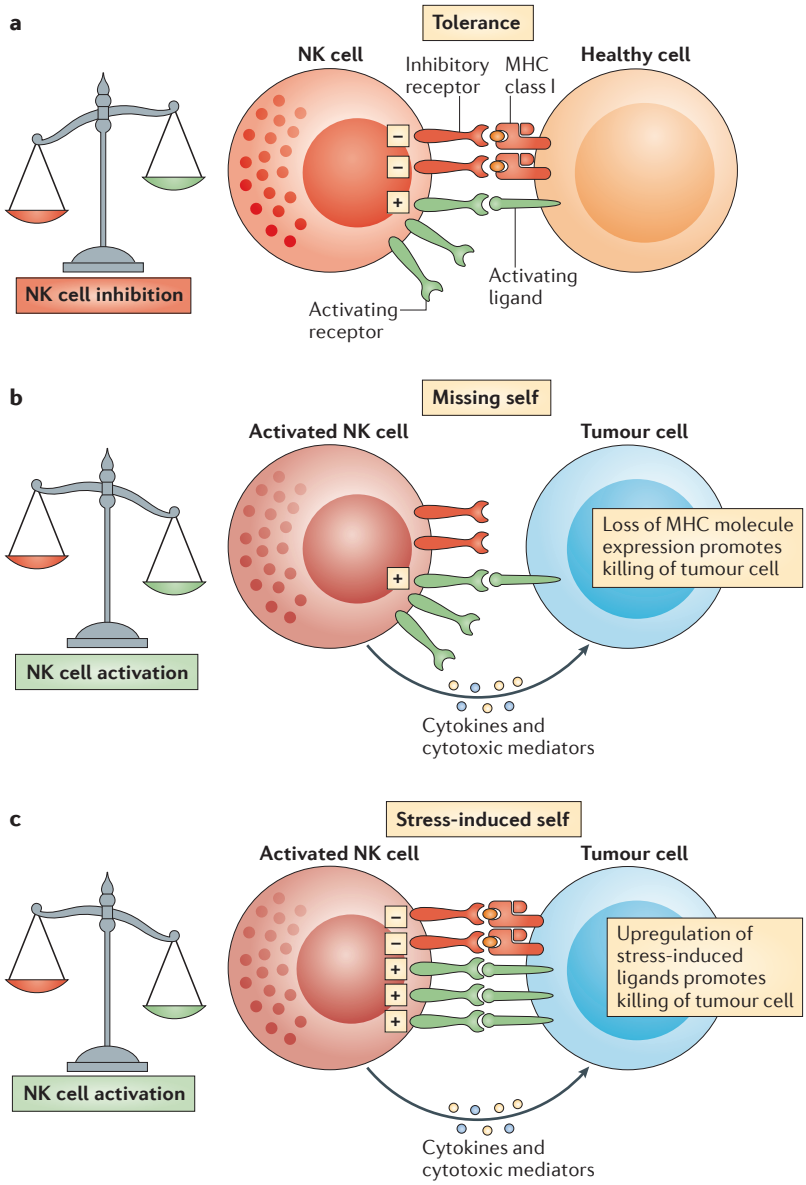

Figure 1 | **Recognition of tumour cells by NK cells. a** | Natural killer (NK) cells are tolerant to healthy host cells, as the strength of the activating signals they receive on encountering these cells is dampened by the engagement of inhibitory receptors (tolerance). **b** | Tumour cells may lose expression of MHC class I molecules. NK cells become activated in response to these cells, as they are no longer held in check by the inhibitory signal delivered by MHC class I molecule engagement. This is known as 'missing-self' triggering of NK cell activation. **c** | In addition, NK cells are selectively activated by 'stressed' cells, which upregulate activating ligands for NK cells and thereby overcome the inhibitory signalling delivered by MHC class I molecules. This is known as 'stress-induced self' triggering of NK cell activation. In both conditions, NK cell activation leads to tumour elimination directly (through NK cell-mediated cytotoxicity) or indirectly (through the production of pro-inflammatory cytokines, such as interferon-γ).

whereas NK cell education is mediated through the engagement of their MHC class I-specific inhibitory receptors. This education, which is also termed 'licensing' or 'arming', leads to the maturation of a functional NK cell repertoire that is adapted to the MHC class I environment of the host[5,53–59]. In MHC class I-deficient individuals, NK cells are hyporesponsive to stimulatory receptor stimulation and thereby tolerant to self[60,61]. This hyporesponsiveness of NK cells grown in an MHC class I-deficient environment can nevertheless be overcome in inflammatory conditions[62]. Thus, two mechanisms of self-tolerance for NK cells coexist in steady-state conditions: first, in functionally competent NK cells, effector responses are inhibited by the recognition of self MHC class I molecules and, second, there are hyporesponsive NK cells that cannot detect self MHC class I molecules.

Of course, this is a simplistic view, and NK cell education does not result in a bipolar situation but rather in a continuum that allows fine-tuning of NK cell responsiveness. For example, NK cells that express higher levels of inhibitory receptors specific for self MHC class I molecules have greater immunoreactivity than NK cells that express lower levels of inhibitory receptors specific for self MHC class I molecules[58,63,64]. The molecular mechanisms underlying this MHC-dependent NK cell education are only partially understood. Nevertheless, a functional immunoreceptor tyrosine-based inhibitory motif (ITIM) is necessary in the intracytoplasmic tail of the mouse LY49 inhibitory receptors[53]. In addition, NK cell education via MHC class I-specific inhibitory receptors does not substantially alter the NK cell transcriptional programme. Rather, it is associated with the confinement of activating receptors in membrane nanodomains. By contrast, these receptors are preferentially located in an actin-rich membrane meshwork in uneducated NK cells[65]. Thus, competent and hyporesponsive NK cells might switch from one state to the other via this mechanism based on the membrane confinement of activating receptors, conferring NK cells with more plasticity than originally thought. Along this line, NK cells expressing inhibitory receptors revert from a hyporesponsive to a competent status following exposure to cognate MHC class I molecules[66,67]. Therefore, following encounter with target cells expressing a low surface density of MHC class I molecules, one could predict that the reactivity of NK cells would decrease as a result of adaptation to this altered MHC class I environment. Despite the recent confirmation of the importance of 'missing-self' recognition for NK cell responsiveness to tumours *in vivo*[68], the kinetics of NK cell adaptation to the surrounding milieu are still unclear but represent a key issue for understanding NK cell reactivity.

Stress-induced self recognition. Besides using inhibitory receptors that recognize self, NK cells are also equipped with cell-surface activating receptors[69,70]. In addition to the recognition of microbial molecules by a variety of innate immune receptors, a process known as 'infectious non-self recognition', it has been shown that several receptors of innate immune cells can detect internal changes that occur in damaged host tissues, leading to the concept of 'stress-induced self recognition'[71–73]. This mode of detection relies on the recognition of self molecules that are barely detectable in steady-state conditions but whose expression increases during various forms of stress.

Box 2 | **NK cells and missing self**

Natural killer (NK) cells use inhibitory receptors to detect the presence of constitutively expressed self molecules on susceptible target cells. In particular, NK cells express MHC class I-specific receptors and are kept in a quiescent state in response to inhibitory signals delivered by these receptors[227–232]. As a consequence, NK cells can recognize 'missing-self' target cells that have downregulated MHC class I expression as a result of infection or transformation[227]. The MHC class I-specific inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) in humans, the lectin-like LY49 dimers in mice and the lectin-like CD94–NKG2A heterodimers in both species[52,230–235]. These inhibitory receptors possess intracytoplasmic inhibitory signalling domains called immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that mediate their inhibitory function[236–238]. Other inhibitory receptors (for example, mouse NKRP1B (also known as KLRB1B), human NKRP1A (also known as KLRB1) and mouse 2B4) that recognize non-MHC self molecules (for example, C-type lectin-related protein B (CLRB), lectin-like transcript 1 (LLT1) and CD48, respectively) also regulate NK cell activation[239].

A prototypical example is the activation of NK cells via engagement of the activating receptor NKG2D. NKG2D interacts with self molecules that are selectively upregulated on stressed cells, such as tumour cells[74–77]. *In vivo*, NKG2D was shown to be crucial for the immunosurveillance of epithelial and lymphoid malignancies in two transgenic models of *de novo* tumorigenesis[78]. In the transgenic Eμ-*Myc* mouse model of spontaneous B cell lymphoma, the expression of NKG2D ligands by tumour cells represents an early step of tumorigenesis that is associated with still-unknown genetic lesions in cancer cells[79]. The ligands for NKG2D are stress-inducible proteins, namely: MICA (MHC class I polypeptide-related sequence A), MICB and members of the ULBP (also known as RAET1) family in humans[71,76] and RAE1 (retinoic acid early-inducible protein 1), H60 and MULT1 (murine ULBP-like transcript 1) in mice[74,75,77]. A link between tumorigenesis, the DNA damage response and the immune response has been proposed. DNA-damaging agents or DNA lesions associated with tumorigenesis activate the DNA damage response in damaged cells. This response results in the upregulation of NKG2D ligands, which stimulate NK cells to attack the diseased cells[80]. The upregulation of NKG2D ligands depends on one of the phosphoinositide 3-kinase (PI3K)-related protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related), which initiate the DNA damage response pathway following the recognition of DNA damage[72]. Treatment with proteasome inhibitors also induces NKG2D ligand expression in multiple myeloma cells via the ATM or ATR pathway[81].

Besides NKG2D, NK cells express an array of other cell-surface molecules, including the natural cytotoxicity receptors (NCRs), which were shown to be involved in the activation of NK cells by tumour cells more than a decade ago. The NCR family includes NKp46 (also known as NCR1 and CD335)[82], NKp44 (also known as NCR2 and CD336)[83] and NKp30 (also known as NCR3 and CD337)[84]. The association of NCRs with signal-transducing polypeptides that contain immunoreceptor tyrosine-based activation motifs (ITAMs) is reminiscent of the architecture of other pivotal immune receptor complexes (such as the TCR, the BCR and Fc receptors) and makes them very potent activating receptors[70]. The tumour cell-expressed ligands for the NCR family have remained elusive, hindering a complete understanding of the role of NCRs in tumour surveillance. An exception resides in the identification of a new member of the B7 family of immunoreceptors, B7-H6, as a cellular ligand for NKp30 (REFS 85,86). The expression of B7-H6 on tumour cells induces NKp30-dependent NK cell activation and cytotoxicity. Importantly, B7-H6 is not expressed by healthy cells in steady-state conditions, but is found on tumour cells. Considering that NK cells do not compromise the integrity of normal healthy cells and tissues, a reasonable hypothesis is that, similarly to NKG2D ligands, self ligands for all activating NK cells receptors are tightly downregulated in healthy cells and are upregulated in stressed cells, such as tumour cells.

There are many other activating receptors and adhesion molecules that are expressed on NK cells and may participate in the recognition of tumour cells. These receptors include DNAM1 (DNAX accessory molecule 1; also known as CD226) — which is activated by its ligands poliovirus receptor (PVR) and nectin 2 (also known as PVRL2)[87] — and the signalling lymphocytic activation molecule (SLAM) receptors 2B4 and NTBA, as reviewed elsewhere[87]. Importantly, both human and mouse NK cells express Fcγ receptor IIIA (FcγRIIIA; also known as CD16), which recognizes antibody-coated target cells through their Fc region and mediates antibody-dependent cellular cytotoxicity (ADCC). Growing evidence indicates that ADCC contributes to the beneficial clinical response of human cancers to several monoclonal antibody-based therapies, including treatment with rituximab[88,89], implying that NK cells are involved in the antitumour response in these settings.

Manipulation of NK cells in tumour therapy

The remarkable conservation of their antitumour activity against many types of mouse and human tumours suggests that NK cells detect common modifications in cellular metabolism and/or gene expression that are shared or induced by many oncogenic processes. This ability of NK cells to target a common mechanism present in cancer cells, while respecting the integrity of healthy cells, has led to them being considered as promising therapeutic tools for cancer immunotherapy[90].

Allogeneic haematopoietic stem cell transplantation for patients with cancer. Allogeneic haematopoietic stem cell transplantation (HSCT) is an efficient form of adoptive immunotherapy in the treatment of patients with haematological malignancies[91]. The therapeutic effect was originally thought to be derived mostly from the escalated doses of cytotoxic agents and radiation therapy used in the so-called 'conditioning regimen'. The reality of a graft-versus-leukaemia effect (GVL effect) induced by the allogeneic graft was nevertheless established more than three decades ago[92]. Since this time, the possibility of disease control in other haematological malignancies[93] and also in solid tumours[94,95] has been confirmed.

T-bet
A member of the T-box family of transcription factors. It is a master switch in the development of T helper 1 (T_H1) cells through its ability to regulate the expression of the IL-12 receptor, to inhibit signals that promote T_H2 cell development and to promote the production of interferon-γ.

Perforin- and granzyme-mediated mechanisms
Granzymes are serine proteases that are found primarily in the cytoplasmic granules of cytotoxic T lymphocytes and NK cells. They enter target cells through perforin pores and then cleave and activate intracellular caspases to induce target-cell apoptosis.

CD95–CD178 pathway
CD178 (also known as FAS ligand) binds to CD95 (also known as FAS). This results in the formation of a death-inducing signalling complex and the subsequent activation of caspases, which promote the apoptosis of the CD95-expressing target cell.

Immunoreceptor tyrosine-based inhibitory motif
(ITIM). A motif that is present in the cytoplasmic domains of several inhibitory receptors. After ligand binding, ITIMs are phosphorylated on their tyrosine residues and recruit lipid or tyrosine phosphatases.

DNA damage response
A cell response triggered by DNA damage, such as single or double strand breaks. The DNA damage response stops cell cycle progression to enable repair before the damage is transmitted to progeny cells. Checkpoints in the mammalian DNA damage response are controlled by the PI3K-related kinases ATM and ATR.

Immunoreceptor tyrosine-based activation motifs
(ITAMs). Activating receptors often have ITAMs consisting of a consensus amino-acid sequence with paired tyrosines and leucines ($Yxx(I/L)x_{6-12}Yxx(I/L)$). These motifs are normally located in the cytoplasmic domains of ligand-binding transmembrane receptors (such as FcεRI and the TCR), and they mediate interactions between the transmembrane receptor complex and protein tyrosine kinases, which are required to initiate early and late signalling events.

Antibody-dependent cellular cytotoxicity
(ADCC). A mechanism used by leukocytes that express Fc receptors to kill antibody-coated target cells.

Rituximab
A chimeric monoclonal antibody that is specific for the CD20 molecule, which is primarily expressed by B cells. Rituximab is the most frequently used antibody therapy for patients with cancer.

Graft-versus-leukaemia effect
The antitumour activity of donor T cells against residual leukaemic cells of the graft recipient following (allogeneic) bone marrow transplantation.

Graft-versus-host disease
(GVHD). Tissue damage in a recipient of allogeneic transplanted tissue (usually a bone marrow transplant) that results from the activity of donor cytotoxic T lymphocytes that recognize the tissue of the recipient as foreign. GVHD varies markedly in severity, but can be life threatening in severe cases. Typically, damage to the skin and gut mucosa leads to clinical manifestations.

Despite these proof-of-concept findings, allogeneic HSCT has only been carried out in a limited subset of patients. This is partly due to the high rate of fatal toxicity attached to the procedure, which eventually impairs patient survival despite effectively controlling disease. These complications have an immunological basis[96]. Indeed, alongside the desirable GVL effect, allogeneic immune activation promotes graft-versus-host disease (GVHD), even when the donor is a fully HLA-identical sibling.

This still represents an important limitation for the development of allogeneic HSCT, along with the lack of donors. Indeed, for years, allogeneic HSCT protocols have failed to manipulate the immune system to boost graft-versus-tumour (GVT) effects without inducing GVHD. To solve this issue, most HSCT protocols have relied on the manipulation of T cell responses, an attempt that proved to be extremely hazardous owing to the underestimated and unpredictable cross-reactivity of the TCRs. By contrast, it is now established in preclinical and clinical settings that NK cells have a unique capacity to exert potent GVT effects without inducing GVHD, a feature that probably results from the differential distribution of ligands for activating NK cell receptors on haematopoietic cells and non-haematopoietic cells (such as epithelial cells)[97].

Allogeneic HSCT has been an evolving field during the last two decades as a consequence of the diversification of stem cell and donor sources and changes in the conditioning regimen. One type of allogeneic HSCT is 'haplo-identical' transplantation, which was developed to overcome the problem posed by a lack of an HLA-matched donor and has recently highlighted again the potential of NK cells in cancer control. In haplo-identical transplantation, the related donor and recipient share only one HLA haplotype. In some but not all cases, the recipient also expresses HLA molecules that are not expressed by the donor, and these HLA molecules are ligands for killer cell immunoglobulin-like receptors (KIRs). In this 'KIR ligand-mismatched' situation, subsets of donor-derived NK cells that are not restrained by host MHC class I molecules develop in the recipient and have GVL potential.

In classical haplo-identical HSCT protocols, the allograft is depleted of T cells before re-infusion to reduce the incidence of severe and potentially lethal GVHD, and the patient is subjected to a highly cytotoxic and immunosuppressive conditioning regimen to prevent graft rejection[98]. In these settings, recipients with acute myeloid leukaemia (AML) who received haplo-identical transplants from KIR ligand-mismatched donors had a marked reduction in relapse rates compared with an otherwise similar group of patients with AML who received transplants from KIR ligand-matched relatives[99]. The positive influence of KIR incompatibility in haplo-identical HSCT was detected in some additional studies in patients with AML[100,101] or multiple myeloma[102], but not in other studies[103], indicating that other parameters in the treatment protocol — such as the dose of stem cells and the extent of T cell depletion — contribute to the clinical outcome. Hence, the spectrum of alloreactivity displayed by donor-derived NK cells remains to be fully elucidated (FIG. 2a).

Clinical efficacy against residual tumour cells requires that fully competent NK cells of donor origin develop in the recipient following transplantation. Considering the role of MHC class I molecules in the education and plasticity of NK cells, as described above, the development of alloreactive NK cells of donor origin is likely to depend on the conditioning regimen administered to the recipient and on the dose of donor haematopoietic progenitors (that is, the likelihood and the duration of interaction with donor or recipient MHC class I molecules). The complexity and changing nature of such interactions is a likely explanation for the apparently inconsistent observations of NK cell alloreactivity in different protocols and studies. The challenge will be to harness the therapeutic potential of NK cells in this context.

NK cells could also be used as part of the conditioning regimen, as it has been shown in mouse models that donor NK cells can impair donor T cell-mediated GVHD by killing host dendritic cells[99]. Irrespective of this possibility, NK cells are especially attractive therapeutic tools because their association with a reduction in relapse rates is not associated with increased GVHD incidence. Although limited, these observations suggest that NK cell manipulation can dissociate GVL and GVHD effects, an objective that has never been attained through the manipulation of T cell compartments. In addition, ciclosporin (also known as cyclosporin A) that is administered to inhibit T cells and prevent GVHD can also induce the expansion NK cell populations and boost NK cell functions[104,105].

Finally, recent work has also focused on the role of activating KIRs (also known as KIR-S) in HSCT for patients with leukaemia. Activating KIRs are homologues of the inhibitory KIRs, but with shorter cytoplasmic tails that are devoid of ITIMs and with a transmembrane domain that associates with the ITAM-bearing signal-transducing polypeptide DAP12 (REFS 106–108). Activating KIRs are potent NK cell activators and can also be expressed by certain rare T cell subsets. The genes encoding activating KIRs are absent in the approximately 25% of Caucasians who are homozygous for KIR gene group A. Investigations into activating KIR genetics have showed that the presence of activating KIRs is associated with lower rates of leukaemia relapse and cytomegalovirus reactivation and improved survival in some patients[109–114]. Thus, several years after the clinical proof of concept for the role of NK cells in HSCT therapy was provided by a pivotal study[99], the question remains as to how NK cell alloreactivity can be fully exploited to produce clinical benefits in haplo-identical HSCT. In addition, these protocols of haplo-identical HSCT remain in use for only a minor subset of patients with poor-prognosis malignancies, because the protocols are associated with a high transplant-related mortality rate owing to profound and durable immunosuppression.

HLA-identical HSCT is still the most frequently used HSCT approach for patients with cancer, and its use continues to increase. The procedure has benefited substantially from the reduced toxicity associated with the new approaches for the conditioning regimen. Following the initial description of the first clinical

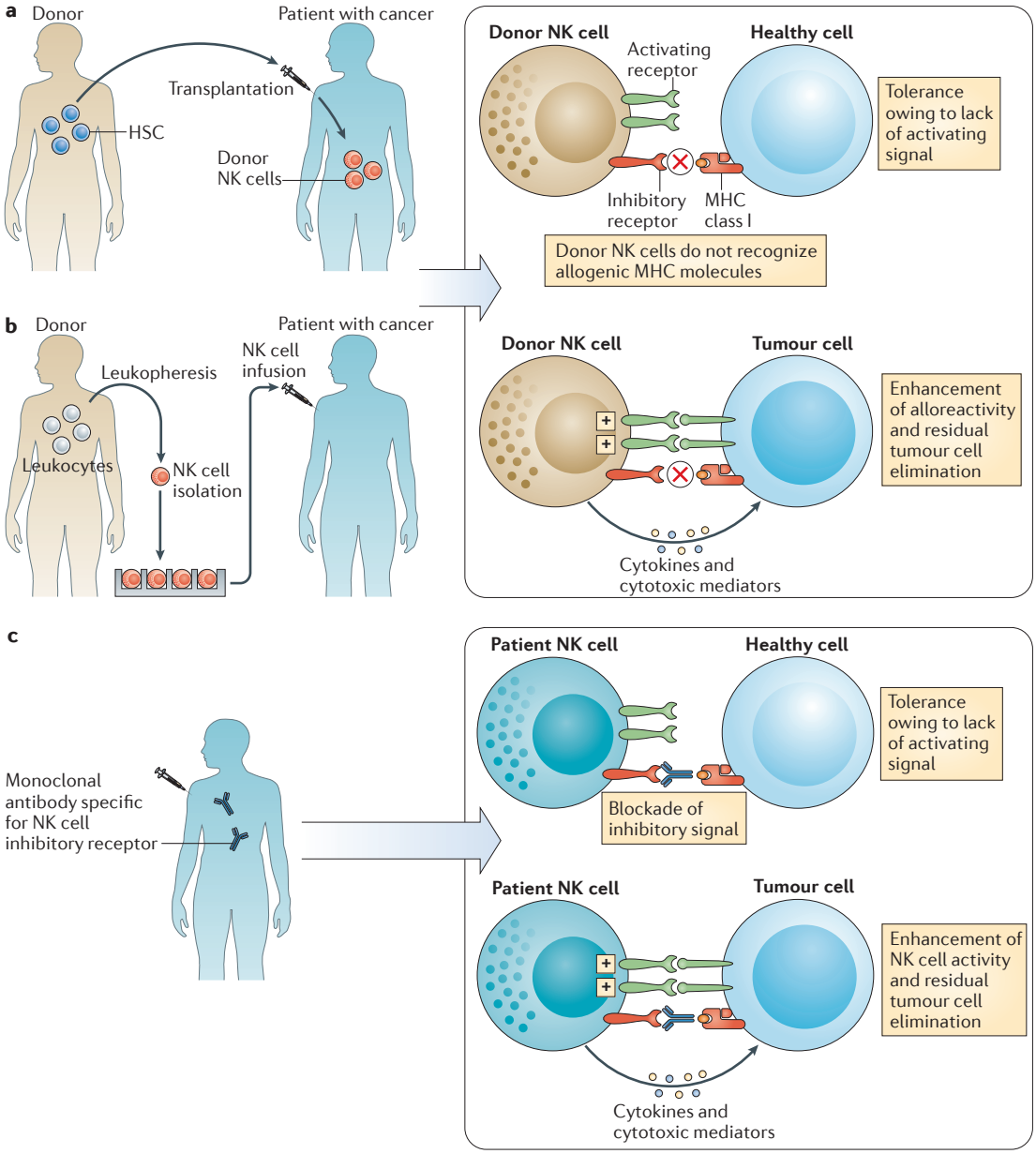

Figure 2 | **Cancer therapies targeting NK cells. a** | Following haplo-identical or MHC-matched haematopoietic stem cell transplantation (HSCT), natural killer (NK) cells of donor origin develop in the patient with cancer. **b** | Alternatively, NK cell populations can be isolated from healthy donors and activated and/or expanded *in vitro* before infusion into the patient with cancer. In both cases (allogeneic HSCT and NK cell infusion), the aim is to promote the antitumour function of donor NK cells in the patient. Indeed, a fraction of donor NK cells will be not be inhibited by the MHC class I molecules of the patient, as the killer cell immunoglobulin-like receptors (KIRs) expressed by the donor NK cells will not interact with the MHC class I molecules of the patient, and this promotes tumour cell elimination. In contrast to cancer cells, most healthy cells of the patient will not activate donor NK cells, as they lack a sufficient cell-surface density of activating ligands for the donor NK cells. **c** | An alternative approach is to boost endogenous NK cell activity by treating patients with monoclonal antibodies specific for NK cell-expressed inhibitory receptors. These antibodies are designed to enhance the antitumour activity of the patient's own NK cells without inducing autoimmunity.

Ciclosporin
(Also known as cyclosporin A). A commonly used immunosuppressive drug that blocks calcineurin A and thereby inhibits T cell activation. It is used to prevent the rejection of transplanted organs and to treat some inflammatory diseases. Ciclosporin is widely used to prevent graft-versus-host disease following allogeneic haematopoietic stem cell transplantation.

success[91], allogeneic HSCT procedures were carried out using basically unmodified protocols for 20 to 25 years. The approach begins with a preparative phase known as conditioning. This phase is followed by infusion of the allogeneic graft cells and post-infusion drug-mediated immunosuppression in an attempt to limit unwanted GVH reactions. The goals of conditioning are to treat residual tumour disease and to allow for donor cell engraftment through the profound immunosuppression of the host. Both goals have been achieved, rather successfully, using a combination of high-dose total-body irradiation with chemotherapy, or using a combination of highly myeloablative and antineoplastic drugs. However, the cytotoxicity of the conditioning regimen

results in the production of pro-inflammatory cytokines, which initiate the previously described negative clinical effects[115]. Indeed, these cytokines enter the blood and activate circulating immune cells from the infused graft, leading to the GVH effects. This now-classical scenario suggested that inhibiting cytokine production could reduce the toxicity of this therapy. This hypothesis was convincingly verified in studies that showed that patients treated with potent immunosuppressive agents during allogeneic engraftment experienced beneficial GVL or GVT effects during allogeneic HSCT, without developing GVHD[116–118].

Since then, these findings have been widely confirmed, and reduced-intensity conditioning regimens (RIC regimens) are now widely used. Moreover, the dramatic decrease in the rates of procedure-related mortality[119] has suggested that allogeneic HSCT could be applicable to a wider population, including patients with diagnoses other than leukaemia (such as lymphoid malignancies and solid tumours) and patients above the age of 50 years, who are normally not considered for transplantation because of their increased risk of developing fatal complications. Indeed, the number of allogeneic HSCTs conducted worldwide has more than doubled in less than 10 years[120].

Altogether, these achievements justify the present attempts to further improve disease control after HLA-identical HSCT and, in particular, to develop approaches that manipulate NK cells. Indeed, donor NK cells are among the first cells to arise in the recipient following allogeneic HSCT[121], with NK cell reconstitution evident as early as 1.5 months following RIC HSCT[122,123]. Various studies have shown an association between higher numbers of NK cells in the graft and lower rates of relapse[114]. High NK cell counts on day 30 following allogeneic HSCT have also been associated with improved clinical outcomes[124,125]. More recently, an association between high NK cell counts at day 60 and reduced relapse after RIC HSCT was reported[126]. This effect was not documented after standard conditioning regimens. Altogether, these data demonstrate that NK cells are present at early time points following RIC HSCT and can exert their functions even during immunosuppressive treatment.

Donor lymphocyte infusions. Nowadays, allogeneic HSCT antitumour activity can be reinforced through single or sequential re-infusions of donor-derived immune-competent cells once haematopoietic chimerism has been established. Initially, in the mid 1990s, donor lymphocyte infusions (DLIs) were shown to result in a high incidence of durable cytogenetic and molecular remissions when used as a treatment for chronic myeloid leukaemia (CML) that relapsed after conventional allogeneic HSCT[127,128]. However, despite these positive effects, significant side effects occurred, including GVHD and secondary aplasia, as a consequence of the high numbers of cytotoxic T cells in the infusions[129]. Attempts to deplete $CD8^+$ T cells from DLIs produced encouraging, although incomplete, results[130,131]. The interest in lymphocyte-driven immunotherapy led to the infusion of allogeneic lymphocytes being explored as a therapy for cancer outside the context of allogeneic HSCT[132]. However, it was noted that lymphocyte survival was only short-term, and this prevented long-term cancer control, although clinical responses were documented in some patients. In line with these initial data, a recent study established that an infusion of HLA-mismatched peripheral blood stem cells improved the outcome of chemotherapy for AML in elderly patients[133]. Whether the use of appropriately selected and activated donor-derived NK cells instead of regular DLIs will better support an antitumour effect remains to be shown, but this warrants further study. Several protocols of clinical-grade NK cell purification and *in vitro* population expansion are now validated, and studies have shown that the infusion of allogeneic NK cells is safe in humans[134–148] (FIG. 2b). Injections of mature HLA-mismatched NK cells are also well tolerated[149]. Therefore, there are encouraging signs that NK cell infusion could be a useful antitumour strategy.

KIR-specific monoclonal antibodies. As already mentioned, the use of allogeneic HSCT remains restricted to minor subsets of patients who are affected by poor-prognosis malignancies. These are mainly patients with acute leukaemia that is associated with poor-prognosis criteria (mostly cytogenetic criteria) and relapse, patients with myelodysplastic syndromes, and patients with lymphoid malignancies who failed to respond or relapsed after initial therapy. Other factors that can restrict the use of allogeneic HSCT include the difficulty in identifying a suitable donor, as well as the physical condition of the recipient and his or her predicted ability to sustain the morbidity associated with the transplantation. As a consequence, allogeneic HSCT can rarely be offered to elderly people, who constitute a population in which the incidence of haematological malignancies is increasing.

In vivo activation of NK cells is an alternative avenue for medical progress that is potentially applicable to a broader group of patients. Fully humanized KIR-specific monoclonal antibodies have been generated to achieve this aim[150,151] (FIG. 2c). By blocking the interactions of all inhibitory KIRs that recognize HLA-C molecules, KIR-specific monoclonal antibodies can boost the reactivity of NK cells against tumour cells that express ligands for activating receptors, without inducing autoimmunity against normal cells, which do not express a sufficient density of activating ligands[152] (FIG. 2c). However, as the recognition of MHC class I molecules by KIRs is crucial for NK cell education, the blocking of KIRs by monoclonal antibodies may have more complex consequences than simply triggering tumour elimination.

To fully evaluate this issue *in vivo*, a humanized preclinical mouse model has been developed, in which all NK cells are educated by a transgenic inhibitory receptor (human KIR2DL3) through engagement with its ligand HLA-Cw3. This approach showed that NK cells could be reprogrammed to kill $HLA\text{-}Cw3^+$ target cells without compromising self-tolerance and without

Reduced-intensity conditioning regimens
Regimens that use less chemotherapy and radiation than is normally used for myeloablation.

abolishing NK cell education[153]. Following preclinical evaluation of KIR-specific monoclonal antibodies, 100 patients with AML or multiple myeloma were treated in Phase I or II clinical studies[154,155]. These studies showed that the infusion of KIR-specific monoclonal antibodies was safe, even in elderly patients who had been heavily pretreated with chemotherapy. Although the results of efficacy are awaited, the availability of KIR-specific monoclonal antibodies paves the way for the design of innovative NK cell-based antitumour therapies. For instance, new protocols might include KIR-specific monoclonal antibodies in combination with HLA-identical or non-identical HSCT, with infusion of NK-selected donor lymphocytes, with other monoclonal antibody cancer therapies (such as CD20-specific or HER2-specific monoclonal antibodies), or with drugs such as lenalidomide that induce the expression of ligands for NK cell activating receptors[156].

In conclusion, the efficacy of NK cell-based tumour therapies has not yet been firmly established. Questions remain regarding the sensitivity of tumour cells to NK cell attack, the migratory properties of endogenous[157] and infused NK cells[158], and the survival and homeostatic proliferative capacity of donor NK cells in patients with cancer in conditions of chemotherapy and/or HSCT[159]. Many clinical trials have been initiated that hopefully will answer some of these key issues (BOX 3).

Roles of NKT cells in cancer

In early studies, the respective functions of NK and NKT cells in antitumour immune responses were sometimes confounded. Recent progress in the characterization of iNKT cells has allowed for a better understanding of their functions in response to tumours. We review below how iNKT cells are thought to recognize tumour cells and how these findings have led to current strategies to target these cells for tumour therapy.

How do NKT cells recognize tumour cells?

The activation of iNKT cells by potent agonists, such as αGalCer, leads to strong antitumour responses in mice (FIG. 3). However, in this case, the iNKT cell contribution to the antitumour response is indirect and mediated in part by downstream effectors, such as NK cells and the cytokine IFNγ, rather than through direct targeting of cancer cells by iNKT cell-mediated cytotoxic mechanisms. iNKT cells can respond directly to IL-12 in the B16 melanoma model and produce IFNγ, but iNKT cells are dispensable for effective antitumour immunity in this model[160]. However, their contribution in the absence of NK cells has not been addressed[161]. The direct recognition of CD1d-expressing tumour cells by iNKT cells has been demonstrated *in vitro* (FIG. 3a). CD1d is expressed on some myelomonocytic leukaemia cells, and it has been shown that these cells are sensitive to lysis by human NKT cells[162]. Similarly, using a CD1d-transfected mouse B cell lymphoma model, it was shown that iNKT cells protect against tumour progression in a CD1d-dependent manner[163]. However, it is unclear how iNKT cells distinguish CD1d expression on malignant cells from CD1d expression on normal cells. It is possible that a different set of self ligands is presented by CD1d on transformed cells, but evidence for this is lacking. Although CD1d expression has been demonstrated on human malignant haematopoietic cells, most solid tumours and cell line models do not express CD1d, or only poorly express CD1d, suggesting an indirect mechanism or cross-presentation to iNKT cells[164,165] (FIG. 3b).

Roles of iNKT cells in tumour immunosurveillance

There are convincing data to suggest a role for iNKT cells in tumour immunosurveillance, at least in mice. A protective role for iNKT cells in tumour immunosurveillance has been demonstrated in various tumour models, including in methylcholanthrene (MCA)-induced fibrosarcomas, in p53 deficiency and in the transgenic adenocarcinoma of the mouse prostate (TRAMP) prostate cancer model[34–37]. These studies were performed in the absence of exogenous stimuli and compared Jα18-deficient and CD1d-deficient mice with wild-type mice. The results suggested that iNKT cells are crucial for tumour immunosurveillance but that type II NKT cells are dispensable. Adoptive transfer experiments using $CD4^{-}CD8^{-}$ iNKT cells from the liver also suggested a key role for this subset in promoting antitumour immune responses[166]. IFNγ production has been shown to be crucial for iNKT cell-mediated antitumour activity, at least against MCA-induced sarcomas[167], and probably promotes NK cell activation. Overall, these studies in mice suggest that iNKT cells have an active role in tumour immunosurveillance and that their absence predisposes to cancer development.

However, the role of NKT cells during MCA-induced carcinoma has been recently questioned[168]. Also, it is not entirely clear how iNKT cells become activated in these models and which CD1d-expressing cells activate them. In addition, the contributions of endogenous lipids and pro-inflammatory cytokines are unknown. In fact, it has been proposed that iNKT cells do not target tumours directly but instead control CD1d-expressing tumour-associated macrophages, thereby preventing these cells from promoting angiogenesis[169] (FIG. 3c). In humans, observational studies have focused mostly on

Box 3 | **NK cell clinical trials**

A survey on the ClinicalTrials.gov database searching for 'clinical trials and NK cells' indicates that more than 200 clinical trials have been registered since 2003. Of these, ~150 are observational studies and ~50 are interventional clinical studies using natural killer (NK) cells (including 20 Phase I studies, 11 Phase I/II studies, 17 Phase II studies and 3 Phase III studies). Various selection and expansion (if any) procedures and clinical situations are represented, demonstrating the current lack of consensus in the field. Most protocols are designed for patients with haematological malignancies, and only 5 trials (10% of the interventional clinical studies using NK cells) recruit patients with solid tumours. In addition, only 7 protocols (14%) are aimed at child patients. Interestingly, 28 trials are designed and conducted in the setting of allogeneic haematopoietic stem cell transplantation (HSCT), 18 of which involve a partially compatible transplant. The other 22 protocols evaluate the use of allogeneic NK cells (obtained mainly from mismatched but related donors) outside the context of regular allogeneic HSCT. Altogether, if completed, these trials will have recruited a total of 1,863 patients. To date, 7 clinical trials have been completed for an initial estimated enrolment of 103 patients, and only 2 of those (NCT00274846 and NCT00354172) have reported results.

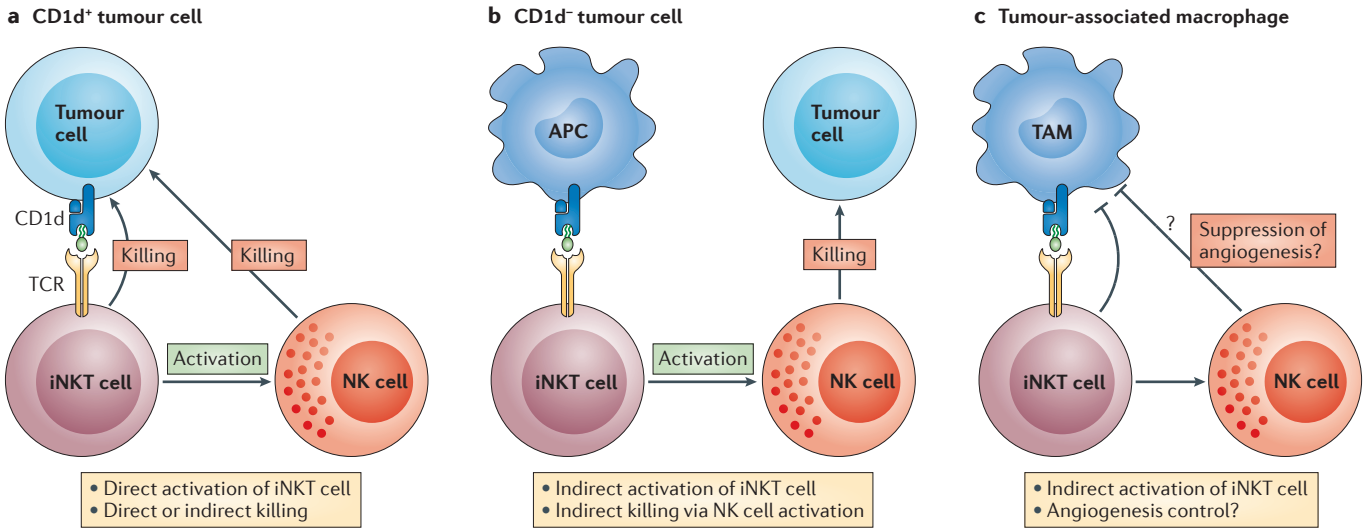

Figure 3 | **Antitumour activities of iNKT cells. a** | Tumour cells that express CD1d can be directly recognized by invariant natural killer T (iNKT) cells and subsequently eliminated either directly, by iNKT cell activity, or indirectly via iNKT cell-mediated activation of natural killer (NK) cells. **b** | Although not all tumour cells express CD1d, iNKT cells can also become activated in response to CD1d-expressing antigen-presenting cells (APCs). The activated iNKT cells then promote NK cell activation and thereby indirectly mediate tumour cell elimination. **c** | It has also been proposed that iNKT cells can limit tumour growth by suppressing the production of pro-angiogenic factors by macrophages, although this still remains to be confirmed. TAM, tumour-associated macrophage; TCR, T cell receptor.

iNKT cells. Overall, iNKT frequency is decreased in solid tumours (including in melanoma and in colon, lung, breast, and head and neck squamous cell carcinomas), and increased iNKT cell numbers are associated with a better prognosis[170–173]. Therefore, these studies in humans are consistent with the mouse studies and suggest that iNKT cells may have a role in tumour immunosurveillance. Interestingly, type II NKT cells have been shown to suppress the tumour immunosurveillance provided by iNKT cells, potentially explaining the paradox in the role of CD1d-restricted T cells in the regulation of tumour immunity[174].

Manipulating iNKT cells for tumour therapy

Initial studies clearly show the antitumour effects of soluble αGalCer in mice[175,166]. Indeed, αGalCer induces rapid iNKT cell activation in mice, and this leads to the downstream activation of NK cells. The cascade of activation initiated by αGalCer-stimulated iNKT cells extends to cells of the adaptive immune system, although this is delayed in comparison to the speed at which iNKT cells promote NK cell activation. However, αGalCer administration can also induce IL-4 production and in some cases anergy in iNKT cells, as the cells become unresponsive to subsequent activation[176,177]. To circumvent these issues, other delivery methods have been examined, including the adoptive transfer of αGalCer-pulsed monocyte-derived dendritic cells, which induce a more-potent antitumour effect than treatment with soluble αGalCer alone (FIG. 4) owing to the superior ability of dendritic cells to present antigens and express co-stimulatory molecules at their cell surface.

A significant amount of work has been undertaken to design glycolipids that will induce a stronger T helper 1 (T_H1)-type immune response and target a specific subset of iNKT cells that are known to promote T_H1-type responses (at least in humans)[178]. Although polarization of the iNKT cell response has been observed using αGalCer analogues, the mechanism leading to this polarization is still under intense investigation[179–181]. Regardless of the mechanism, it was shown that the immediate iNKT cell response is not polarized[182].

Two groups targeted mouse CD1d itself and showed that injection of a CD1d-specific monoclonal antibody has the capacity to promote the maturation of antigen-presenting cells, leading to the production of pro-inflammatory cytokines and the prevention of tumour growth[183,184]. However, the CD1d-mediated intracellular signalling pathway has not been defined, and it is not known whether human CD1d can perform similar functions to mouse CD1d. Notably, a recent study showed that blocking antibodies specific for CD1d had opposite effects and increased tumour metastasis[185]. Targeting specific CD1d-expressing cell subsets has been attempted using antigen–αGalCer conjugate particles preferentially adapted for B cells or CD169+ macrophages[186,187]. Another strategy involved expanding autologous iNKT cell populations *in vitro* to compensate for the decreased iNKT cell frequency observed in patients with cancer[188,189]. Clinical trials using αGalCer alone or αGalCer-pulsed antigen-presenting cells showed that these treatments are reasonably safe and well tolerated. The administration of soluble αGalCer did not result in significant clinical benefits[190,191], although injection of αGalCer-loaded

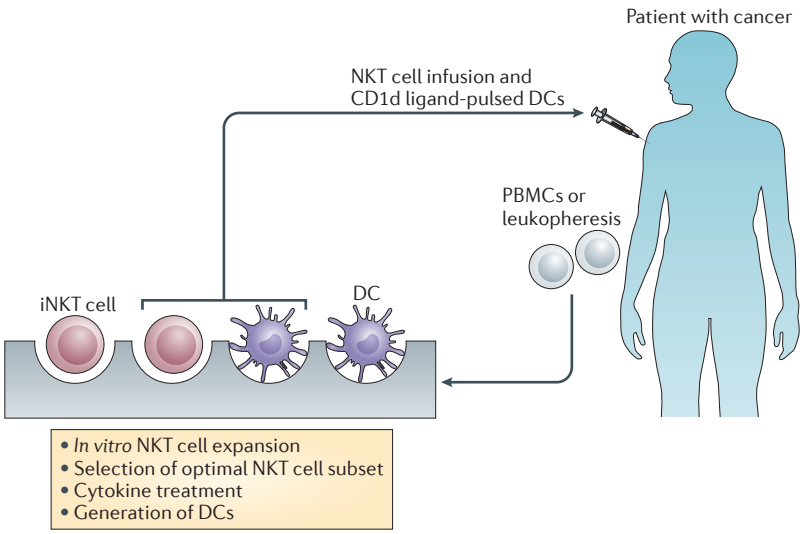

Figure 4 | **Targeting iNKT cells for cancer therapy.** Invariant natural killer T (iNKT) cell populations are isolated from a patient with cancer and expanded *in vitro* before being infused back into the patient. The expanded iNKT cell populations are co-infused with CD1d ligand-pulsed dendritic cells (DCs) to enhance the antitumour activity of the iNKT cells *in vivo*. PBMC, peripheral blood mononuclear cell.

immature dendritic cells led to modest iNKT cell activation *in vivo*[192,193]. However, when mature monocyte-derived dendritic cells were used, iNKT cell population expansion was observed, and this led to an increase in serum levels of IL-12 and IFNγ[194]. Interestingly, the combined transfer of iNKT cells and αGalCer-pulsed dendritic cells has been reported to induce substantial antitumour immunity in patients with head and neck squamous cell carcinomas[189,195] (FIG. 4).

Overall, there is strong evidence for iNKT cell roles in tumour immunosurveillance and for the antitumour potential of ligand-activated iNKT cells. Therefore, additional studies are warranted to optimize glycolipid delivery and to target specific iNKT cell subsets.

Perspectives on NK and NKT cell antitumour roles

Recent studies indicate that NK cell activation could lead to the generation of 'memory' NK cells, a feature that has been ascribed only to B and T cells so far and that is recognized as the hallmark of the adaptive immune system. In the model of MCMV infection, memory-like NK cells have increased reactivity not only to molecules that activate their MCMV receptor, LY49H, but also to NK1.1 (also known as KLRB1C)[42]. Similarly, NK cells that have been pre-activated by cytokines, such as IL-12 and IL-18, are more-readily activated in response to NK1.1 or LY49H stimulation[196]. In addition, hapten- and virus-specific memory NK cells have been described[197,198]. As a consequence of the expression of activating receptors involved in tumour elimination (for example, NKp46, NKp30 and NKG2D) on most if not all NK cells, NK cells that have experienced a non-tumour-driven mode of activation (for example, in response to infection or adjuvants) might display broad cross-reactivity and thus exert better antitumour function, as shown many years ago[199]. The capacity to produce primed or memory NK cells to fight against cancer, as well as the possibility of driving the expansion of tumour-specific memory NK cell populations, thus represents an attractive avenue to explore.

αGalCer has been tested as a potential adjuvant owing to its ability to induce the activation of various immune cells, including NK cells, in both mice and humans[200–207]. These and other findings led to the development of αGalCer analogues as well as strategies to better exploit iNKT properties in the design of adjuvants or vaccines[208–211]. Therefore, antitumour therapies that take advantage of the adjuvant potential of iNKT cell ligands and the effector functions of both NK and iNKT cells warrant future investigations.

Finally, detrimental roles of NK cells have recently emerged in conditions of inflammation (as NK cells can aggravate sepsis)[212], during autoimmunity (as they might contribute to the onset of pathology and to tissue damage)[213], and during microbial infections (as they can dampen subsequent T cell responses)[214,215]. As inflammation is now recognized as a key element in tumour development[216], and antitumour T cell functions have been shown to increase patient survival[217], these issues should be considered when establishing robust immunotherapy protocols. It is also essential to improve immune monitoring in the blood and tissues to explore the role of NK and NKT cells during cancer in large cohorts of patients.

Our knowledge of antitumour immune control has recently progressed rapidly, and a new vision of immunotherapy has emerged from new concepts, medical strategies, medications and medical devices. It is likely that in the coming years the reciprocal movement from bench to bed and from bed to bench will continue to accelerate both the expansion of scientific knowledge and the development of innovative treatments. In this regard, NK cells and NKT cells represent very exciting potential targets for tumour immunotherapy.

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Acknowledgements
E. V. and S. U. are supported by funds from a THINK European Research Council advanced grant, the Agence Nationale de la Recherche and the Ligue Nationale Contre le Cancer, and by institutional grants from INSERM, CNRS and the Université d'Aix-Marseille to the Centre d'Immunologie de Marseille-Luminy. E.V. is a scholar of the Institut Universitaire de France, and a co-founder of and shareholder in Innate Pharma. D.B. and C.C. are supported by grants from the Institut Paoli-Calmettes, the Programme Hospitalier de Recherche Clinique (PHRC) and the Association pour la Recherche sur le Cancer. L.B. is supported by US National Institutes of Health research grants AI46709 and AI058181.

Competing interests statement
The authors declare competing financial interests: see Web version for details.

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