REVIEW

Invariant Vα14 NKT lymphocytes: a double-edged immuno-regulatory T cell population

Mariette Lisbonne and Maria C. Leite-de-Moraes*

CNRS FRE 2444, Paris V, Hôpital Necker, 161 rue de Sèvres, 75743 Paris Cedex 15, France

*Corresponding author: M*aria* C. L*eite-de-*M*oraes*, CNRS FRE 2444, P*aris* V, H*pital* N*ecker*, 161 *rue de* S*vres*, 75743 P*aris* C*edex* 15, F*rance*.
T*el*: +33 1 44 49 53 94, F*ax*: +33 1 44 49 06 76.
E-*mail*: *leite.de.moraes@necker.fr*

INTRODUCTION

The term "natural killer T (NKT)" cells has initially been coined to designate T lymphocytes expressing NK markers, such as NK1.1 (NKR-P1A or CD161). Later on, this population turned out to be quite heterogeneous and it is now well established that it comprises several subsets with distinct functional capacities. The first evidence for NKT cells came from the work of Fowlkes *et al*. and Budd *et al*. [1, 2] who identified in 1987 a small population of mature $CD4^-$ $CD8^-$ double negative (DN) thymocytes, which differed from conventional T cells by their preferential expression of Vβ8. This unusual subset displayed CD44, CD5 and NK1.1 surface markers [3] and had a $CD4^+$ counterpart with similar phenotypic features [4]. Both DN and $CD4^+$ NKT thymocytes produced Th1 as well as Th2 cytokines [5-7] and were preferentially expanded in response to IL-7 [8]. At the time these data were reported, a study of suppressor T cell lines by Taniguchi's group [9, 10] revealed that most of these expressed the NK1.1 marker and a single invariant Vα14 TCR consisting of the *Vα14* and *Jα281* gene segments with a single-nucleotide N-region. The link between these cell populations was finally established by the work of Lantz & Bendelac [11] who demonstrated that $V\beta8^+$ $CD5^{high}$ $CD44^+$ $CD4^+$ $CD8^-$ or $V\beta8^+$ $CD5^{high}$ $CD44^+$ $CD4^-$ $CD8^-$ thymocyte hybridomas expressed mRNA for the invariant Vα14 TCR. These authors identified also the human equivalent of Vα14 cells, the Vα24-JαQ NKT population [11]. Because the tools for a direct analysis of the invariant Vα14 NKT cells became available only recently with the development of CD1d tetramers, the term NKT cells was initially assigned to all lymphocytes displaying the NK1.1 (NKR-P1A or CD161) marker or even CD44, CD122 or Ly49 molecules, in mouse strains, which did not express NK1.1 [12, 13]. These studies provided important clues to the potential roles of NKT cells. Yet, because of the inherent heterogeneity of $NK1.1^+$ TCR^+ lymphocytes, the lack of specific markers capable of readily discriminating between the different subpopulations has hampered the understanding of their distinct immunoregulatory functions.

Invariant Vα14 NKT cells

*i*Vα14 lymphocytes constitute the major subset of NKT cells, characterized in mice by a rearrangement of the variable region Vα14 to the joining region Jα18 (formerly Jα281) to form an invariant complementarity-determining region 3α (CDR3α) [10, 11]. This single Vα TCR is preferentially associated with Vβ8.2, Vβ7 or Vβ2 [3, 11]. In humans, the invariant Vα24-Jα18 (formerly JαQ) is co-expressed with Vβ11 [14, 15].

Both mouse *i*Vα14 and human *i*Vα24 lymphocytes are positively selected by the non-classical class I molecule CD1d [15-17]. Consequently, *i*Vα14 cells are absent in both $\beta_2m^{-/-}$ and $CD1d^{-/-}$ mice [18, 19]. In contrast to conventional T cells, *i*Vα14 hybridomas and *i*Vα24 clones can be stimulated by CD1d transfectants, suggesting that these cells present an overt or latent autoreactivity to CD1d molecules [14, 20]. The natural ligand of these cells is still unknown, but the glycolipid α-galactosyl-ceramide (α-GalCer), first purified from marine sponges on the basis of its anti-tumor activity, is presented by CD1d molecules and specifically recognized by *i*Vα14 and *i*Vα24 lymphocytes [14, 21, 22]. Taking advantage of these features, two groups have recently produced tetramers of the CD1d molecule loaded with α-GalCer [23, 24], which makes possible a complete and quantitative clonal description of the major NKT cell populations based on their antigen specificity.

Using α-GalCer/CD1d tetramers, it could be demonstrated that *i*Vα14 T cells in C57BL/6 mice usually express NK1.1 and other NK receptors [13, 23-25]. Yet, the expression of these two markers does not completely overlap. Indeed, most tetramer-positive cells in thymus, liver, and bone marrow are also $NK1.1^+$. Conversely, $NK1.1^+$ T cells are not always tetramer-positive, as exemplified by $NK1.1^+$ tetramer-negative T cells in bone marrow and spleen, especially among the DN population [23]. These results clearly demonstrate the presence of minor subsets of $NK1.1^+$ $V\alpha14^-$ NKT cells. The use of α-GalCer/CD1d tetramers confirmed that *i*Vα14 T cells express CD69, CD44 and CD5, but not DX5, a common marker of classical NK cells [13, 23, 24].

Minor NKT cell subsets

CD1d-dependent cells expressing a more diverse TCR repertoire, without being polyclonal, represent a minor subset of NKT cells in mice [20, 26-31]. They belong mainly to the $CD8^+$ or DN phenotype, are absent in $CD1d^{-/-}$ but not in $J\alpha18^{-/-}$ mice, which are exclusively deficient in the *i*Vα14 NKT cell.

A subset of $NK1.1^+$ $TCR\alpha\beta^+$ lymphocytes was also identified in CD1d-deficient mice. This CD1d-independent NKT population, can be $CD8^+$, $CD4^+$ or DN and is preferentially located in spleen and bone marrow rather than in thymus and liver [26-28, 32-34]. As NK1.1 (or CD161) can be expressed upon activation of conventional T cells [33, 34], it is possible that CD1d-independent $CD161^+$ $TCR\alpha\beta^+$ cells belong to a subset of conventional memory T cells. In support of this idea, it was reported that the frequency of human $CD161^+$ $TCR\alpha\beta^+$ T cells is much higher than that of *i*Vα24 T cells [35].

In this review, we focus on the immuno-regulatory functions of *i*Vα14 NKT cells and on recent findings demonstrating their capacity to regulate both Th1 and Th2 immune responses.

DEVELOPMENT OF iVα14 NKT CELLS

It is still controversial whether *i*Vα14 NKT cells originate exclusively from the thymus or not [36, 37]. Indeed, $NK1.1^+$ TCR^+ lymphocytes were detected in nude mice. However, in young athymic animals these cells were α-GalCer/CD1d tetramer-negative in the spleen as well as in the liver [38]. Recent studies using FTOC (fetal thymus organ culture) clearly confirmed that *i*Vα14 NKT cells can develop in the thymus in the absence of extrathymic factors, but did not exclude an alternative, extrathymic differentiation pathway [39, 40].

Several studies indicate that the maturation of *i*Vα14 NKT cells is essentially different from that of conventional T cells. In contrast to the latter, which are positively selected by thymic epithelial cells, *i*Vα14 NKT undergo a positive selection through CD1d-expressing cortical $CD4^+$ $CD8^+$ DP (double positive) thymocytes [41]. Several gene mutations affect *i*Vα14 NKT differentiation rather than conventional T cells. Thus, NKT cell development is impaired or absent in mice deficient for $\beta_2$microglobulin, CD1d, Fyn (a Src protein tyrosine kinase), Ets or EBI-3 (Epstein-Barr virus-induced gene 3) [18, 19, 42-44]. Furthermore, their number is greatly reduced in mice lacking IL-7, IL-7R, IL-15R, GM-CSFRβ (granulocyte/macrophage colony-stimulating factor receptor β), IRF (interferon-regulatory factor-1), lymphotoxin (LT), LTβ receptor or endosomal protease cathepsin L [45-50].

Until recently, it was impossible to characterize *i*Vα14 NKT precursors unequivocally. Therefore, the first *i*Vα14 T cells to appear during ontogeny were identified as $NK1.1^-$ HSA^{low} $CD44^{low}$ Vα14 T cells only a short time ago by two groups who employed α-GalCer/CD1d tetramers for this study [39, 40]. In contrast to mainstream T cells, these *i*Vα14 NKT precursors expand massively at the mature HSAlow stage. Interestingly, the expression of the NK1.1 marker is a late event, correlating with cessation of cell division and establishment of long-term residence either in the thymus or after emigration into peripheral tissues. Another important finding of these studies was that the cytokine profile of freshly sorted *i*Vα14 precursors changes as they progress through the $CD44^{low}$ $NK1.1^-$, $CD44^{high}$ $NK1.1^-$, and $CD44^{high}$ $NK1.1^+$ differentiation stages, from the production of IL-4 alone, IL-4 (high) plus IFN-γ (low), and IL-4 (low) plus IFN-γ (hight), respectively [40]. These data provide further insights into developmental pathways, which might be involved in the expansion of autoreactive cells and in their differentiation into regulatory cells.

ROLES OF iVα14 NKT CELLS

The *i*Vα14 NKT cells have unique functional capacities. In notable contrast with conventional T lymphocytes, they promptly produce large amounts of both IL-4 and IFN-γ upon specific TCR stimulation after *in vivo* administration of α-GalCer [51-53]. Other cytokines, such as IL-3, IL-10, IL-13, GM-CSF and TNF-α might also be produced in these conditions [53, 54] (Figure 1). The final effect of *i*Vα14 NKT cells on other cell populations of the innate and acquired immune system, namely B and T lymphocytes, NK cells, dendritic cells or granulocytes [52, 55-60] depends on the cytokine profile.

What are the mechanisms leading to the generation of the particular cytokine pattern that will determine the immuno-regulatory properties of *i*Vα14 NKT cells ? Recent results suggest that the production of IL-4 or IFN-γ by *i*Vα14 NKT cells might be influenced by the type of antigen-presenting cells they encounter as well as by their microenvironment [61-63]. In support of this idea, we have previously demonstrated that some cytokines can preferentially enhance the production of IL-4 by *i*Vα14 NKT cells, while others favor the secretion of IFN-γ (Figure 2). Indeed, both IL-7 and IL-18 increase the capacity of *i*Vα14 NKT cells to produce IL-4, while IL-12 stimulates the synthesis of IFN-γ [45, 52, 64, 65]. It is noteworthy that IL-4 is exclusively produced in response to TCR cross-linking. Yet, we have recently shown that *i*Vα14 NKT cells can also be fully activated in the absence of TCR CD3 engagement, when they are exposed to the pro-inflammatory cytokine IL-18 together with IL-12 [66] (Figure 3). In this case, high levels of IFN-γ are generated in the absence of IL-4. These results support the notion that *i*Vα14 NKT cells can participate in acquired as well as innate immune responses in an antigen-independent manner.

It is still unclear whether the $CD4^+$ DN subsets of *i*Vα14 NKT cells share the same functional properties upon activation. In mice, both $CD4^+$ and DN peripheral *i*Vα14 NKT cells can produce IL-4 and IFN-γ, even though $CD4^+$ cells are apparently the main producers [23, 53]. Recently, we have described a subset of $CD4^+$ *i*Vα14 NKT cells, which expresses CD1d molecules and auto-presents the α-GalCer [67]. The cytokine production of these cells is characteristically biased towards IL-4. Two *i*Vα24 NKT populations were recently reported in freshly isolated peripheral blood, a $CD4^-$ subset that selectively produced Th1 cytokines (IFN-γ, TNF-α) and a $CD4^+$ fraction which produced both Th1 and Th2 cytokines efficiently [25, 68, 69]. This clear-cut difference in the cytokine profile generated by $CD4^+$ and $CD4^-$ tetramer-positive cells, even when they were stimulated

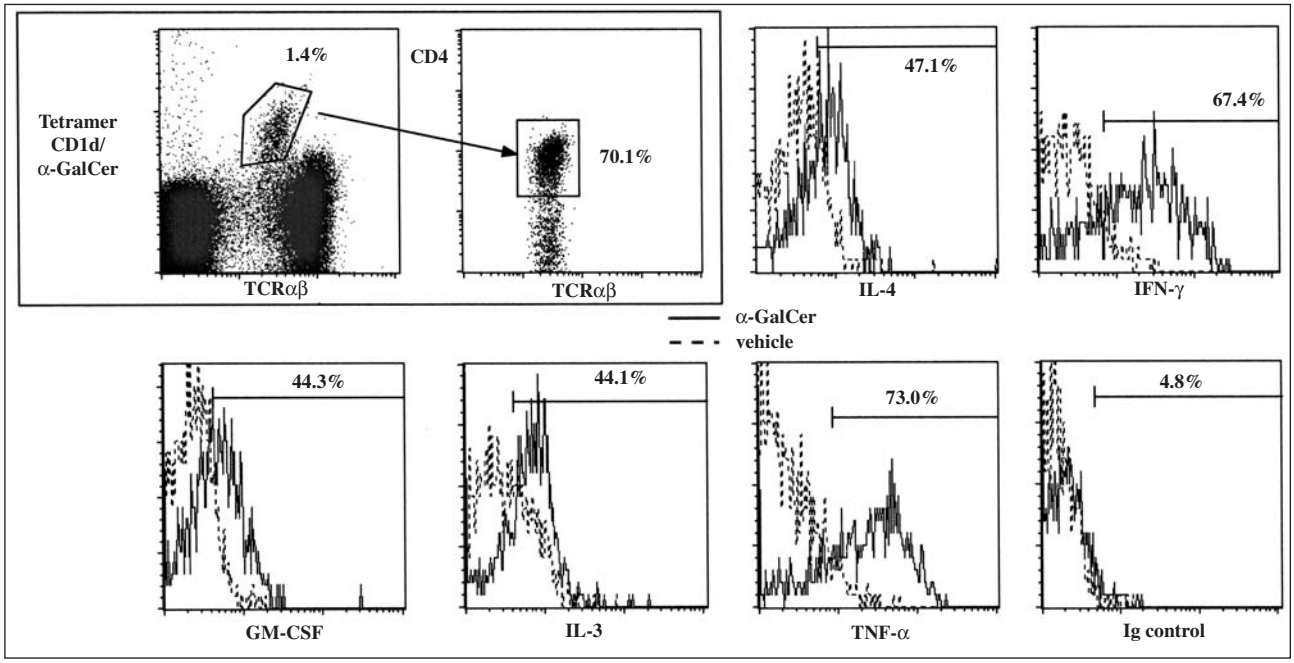

Figure 1

In vivo **activated *i*Vα14 NKT cells produce IL-3, IL-4, IFN-γ, GM-CSF and TNF-α. NKT cell-enriched spleen populations from C57BL/6 mice having received 90 min before 2 µg of α-GalCer or vehicle were stained with anti-TCRαβ, CD1d/α-GalCer tetramers, anti-CD4 and the relevant anti-cytokine mAbs. First two panels: lymphocyte gate defining the TCRαβint CD1d/α-GalCer tetramerspos CD4pos population. Other panels: staining with mAbs against IL-4, IFN-γ, GM-CSF, IL-3, TNF-α. or isotype control among gated lymphocytes.**

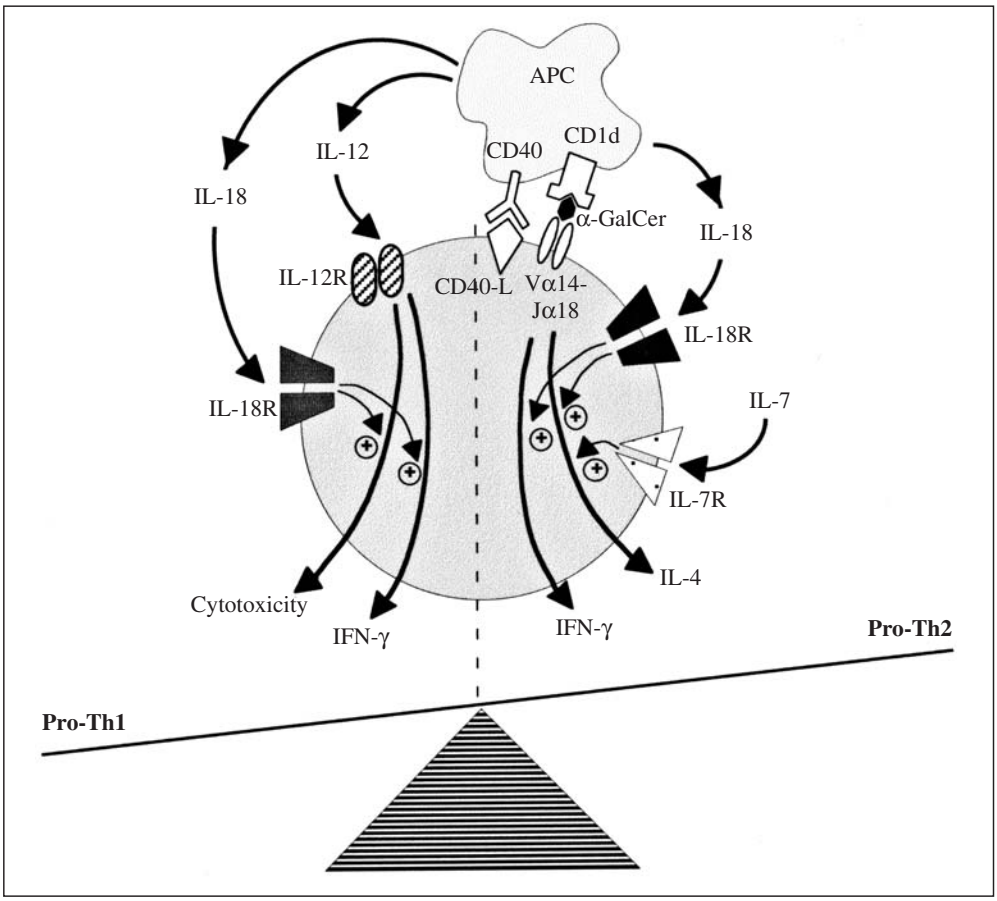

Figure 2

The microenvironment influences *i*Vα14 NKT cell functions. Both IL-7 and IL-18 amplifies IL-4-producing capacity of *i*Vα14 NKT cells while IL-12 enhances their IFN-γ secretion following TCR cross-linking. In response to IL-12 associated to IL-18, *i*Vα14 NKT cells produce IFN-γ without IL-4.

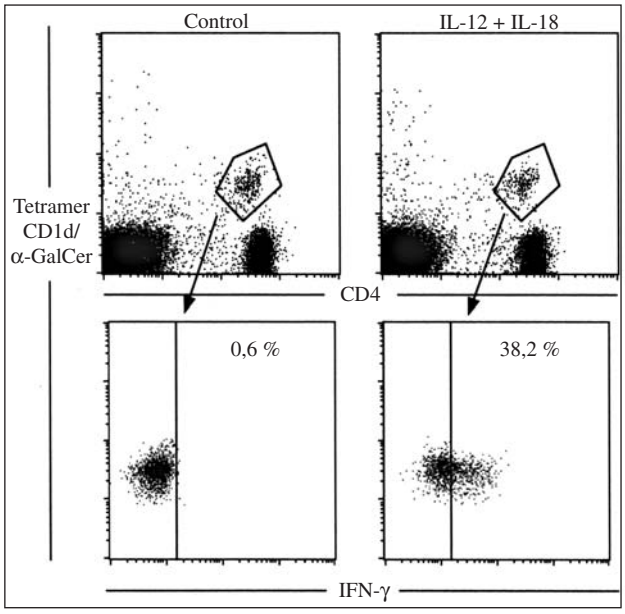

Figure 3
***In vivo* IL-12 plus IL-18 activated *i*Vα14 NKT cells produce IFN-γ. NKT cell-enriched spleen populations from C57BL/6 mice having received 90 min before 1 µg of IL-18 plus 0.2 µg of IL-12 or control were stained with CD1d/α-GalCer tetramers, anti-CD4 and the anti-IFN-γ. Upper panels: lymphocyte gate defining the CD1d/α-GalCer tetramers-pos CD4pos population. Lower panels: staining with mAb against IFN-γ among gated lymphocytes.**

by PMA plus ionomycin or α-GalCer, strongly suggests that they play a distinct role during the immune response.

Other mechanisms may influence the cytokine profile of *i*Vα14 NKT cells. According to a recent report, a more prolonged IFN-γ production can be obtained when *i*Vα14 NKT are stimulated with α-GalCer-loaded dendritic cells rather than with α-GalCer alone [62]. On the other hand, exposure to an analogue of α-GalCer with a truncated sphingosine chain [70] seems to induce a preferential increase in IL-4 production.

In addition to their capacity to produce a variety of cytokines, *i*Vα14 NKT cells exert an important cytotoxic activity [59, 64-66, 71]. According to some reports, target cell lysis *in vitro* might involve the perforin pathway [72]. Yet, we found that *i*Vα14 NKT lymphocytes activated *in vivo* by α-GalCer or IL-12 plus IL-18 could also kill their targets in a Fas/Fas ligand-dependent manner [59, 66]. In humans, a large percentage of $CD4^-$ *i*Vα24 NKT cells is primed for its cytolytic function after exposure to IL-2 and IL-12 [25], suggesting their preferential implication in cytolytic antibacterial, antiviral and anti-tumor immune responses.

It is plausible that the potential auto-reactive effector functions of *i*Vα14 NKT cells require a strict surveillance, as far as their functional capacities, which ensure a prompt riposte during the early stages of the immune response, may become harmful thereafter, causing damage to the organism itself. Indeed, we have demonstrated that NKT cells can be stimulated by IL-12 plus IL-18 or TCR ligation to rapidly initiate effector functions and then, within hours, to undergo activation-induced cell death (AICD) [73]. This mechanism which controls the life-span of activated *i*Vα14 NKT cells is Fas-dependent.

IMMUNO-REGULATORY ROLES OF iVα14 NKT CELLS

The spectrum of actions attributed to *i*Vα14 NKT cells is particularly diverse. In account of their potential auto-reactivity, many studies have suggested that their natural function might be that of suppressor cells protecting self-tissues from damaging inflammatory-type immune responses, such as those resulting in autoimmunity. On the other hand, *i*Vα14 NKT cells are also potentially implicated in controlling immune responses against infection and tumors. These immunoregulatory roles of *i*Vα14 NKT cells, detailed below, are closely related to their cytokine-producing capacities.

iVα14 NKT cells and peripheral tolerance

An experimental model of systemic tolerance termed "Anterior Chamber-Associated Immune Deviation" (ACAID), has provided new insights into the essential role of *i*Vα14 NKT cells in the differentiation of T regulatory (Tr) cells [74]. It was demonstrated that IL-10, potentially produced by *i*Vα14 NKT cells, is critical for the generation of the Ag-specific Tr cells and systemic tolerance in this model [75]. The colocalization of *i*Vα14 NKT cells and $CD1d^+$ tolerogenic APCs, probably a subpopulation of marginal zone (MZ) B cells [76], in the spleen is a requisite for the generation of $CD8^+$ Tr cells. The major signals involved in NKT cell and APC recruitment to the spleen seem to be provided by the chemokines macrophage-inflammatory protein-2 (MIP-2) and RANTES, respectively [77, 78]. In the absence of high-affinity receptors for MIP-2 (as in CXCR2-deficient mice) or by blocking MIP-2 or RANTES by their respective antibodies, peripheral tolerance is actually prevented, and Ag-specific T regulatory cells are not

generated. The implication of *i*Vα14 NKT cells in this model of tolerance is well defined, but the underlying mechanisms need to be elucidated.

iVα14 NKT cells and Th2-mediated inflammation

In account of their capacity to produce rapidly high levels of IL-4, it has been hypothesized that *i*Vα14 NKT cells are required for the development of Th2 immune responses [6, 7]. Most investigations performed with NKT-deficient mice do not support this hypothesis [18, 19]. Nevertheless, they do not exclude the participation of *i*Vα14 NKT cells in some Th2-associated responses. For example, Vα14 TCR transgenic mice, which have increased numbers of NKT cells, as well as α-GalCer-treated wild-type controls, have elevated IgE and IL-4 levels in their serum [51, 79-81]. Furthermore, their *in vivo* activation effectively promotes Th2-associated immunity [70, 81, 82].

The capacity of *i*Vα14 NKT cells to influence immune responses potentially through their IL-4 production was recently supported by a model of contact sensitivity (CS) responses and experimental allergic asthma. CS responses are the prototype for a variety of delayed-type hypersensitivity (DTH) reactions, characterized by the recruitment of effector T lymphocytes from the circulation into peripheral tissues. Askenase [83] demonstrated that IL-4-producing NKT cells are critical for the activation of B-1 cells, resulting in a rapid (24 hours) production of IgM. The latter will form complexes with the challenging antigen, which will locally activate complement and lead to vascular activation, T cell recruitment and CS responses.

In accordance with these finding we have recently demonstrated that *i*Vα14 NKT cells can mediate Th2 immune responses in an experimental model of allergen-induced asthma [60]. Indeed, in *i*Vα14 NKT cell-deficient mice ($J\alpha18^{-/-}$) OVA-specific IgE levels and both IL-4 and IL-5 production were decreased in bronchoalveolar lavage fluid (BALF). Furthermore, the development of eosinophilia and hyperreactivity was impared in comparison with wild-type mice. These hallmarks of allergic asthma were blocked when controls were treated with anti-CD1d. Our findings indicate that *i*Vα14 NKT cells mediate Th2-induced airway inflammation to allergen antigens through a CD1d-dependent mechanism and represent the first demonstration of an endogenous activation of these cells during an antigen-specific immune response.

In addition to IL-4, *i*Vα14 NKT cells can mediate inflammation through an IL-13-dependent mechanism. IL-13 is the major pathologic factor in experimental oxazolone colitis (OC), which shares many histological features with human ulcerative colitis. Colitis cannot be induced in either CD1d- and Jα18-deficient mice, showing the major role of *i*Vα14 NKT cells in this model [84]. Even though these cells can effectively produce IL-13 in response to α-GalCer, it remains to be established that they are directly responsible for the production of this cytokine in the OC model.

iVα14 NKT cells and autoimmunity

Quantitative and functional impairments of *i*Vα14 NKT cells have been reported in some autoimmune conditions such as type 1 diabetes or experimental autoimmune encephalomyelitis (EAE), although their precise implication in the development or severity of these diseases is still unclear.

Type 1 diabetes

In terms of autoimmunity, the most conclusive data concerning the role of *i*Vα14 NKT cells were obtained in diabetes-prone non-obese diabetic (NOD) mice, the experimental model of type-1 autoimmune insulin-dependent diabetes mellitus (IDDM). Type 1 diabetes in NOD mice may be favored by immune dysregulation leading to the hyporesponsiveness of regulatory T cells and activation of effector diabetogenic Th1 cells. A defect in the number and function of *i*Vα14 NKT cells NOD mice was first reported by Gombert *et al*., suggesting that these cells participated in the development of the disease [12]. Similarly, human diabetic patients were found to have lower frequencies of DN $V\alpha24J\alpha18^{+}$ T cells in peripheral blood than their nondiabetic siblings [85]. However, despite marked differences in the percentage of *i*Vα24 NKT cells between individuals, a recent contradictory report showed that *i*Vα24 NKT cell frequency and IL-4 production are similar in IDDM patients and healthy controls, including discordant twin pairs [86]. The major problem hampering the study of *i*Vα24 NKT cells in humans is their heterogeneity and the low incidence of CD1d/α-GalCer-positive cells in peripheral blood (ranging from 0.001 to 0.1%) even in healthy individuals [25, 86, 87]. This means that large cohorts need to be analyzed to answer the question whether the severity of the disease in IDMM patients correlates with modifications in the *i*Vα24 NKT cell compartment, and to address the mechanism of action of these cells.

In the experimetal murine model, both adoptive transfer experiments [88, 89] and forced expression of a *i*Vα14 transgene in NOD mice led to increased numbers of α-GalCer- and CD1d-reactive cells and partial protection from diabetes [79]. When NOD mice bred on a $CD1d^{-/-}$ genetic background were used, increased frequency of these cells and earlier onset of disease were observed in two cases [90, 91], but not in a third one [82]. This discrepancy might be explained by the fact that fewer back-crosses to the NOD background were carried out in the latter occurrence. Further studies using $J\alpha18^{-/-}$ NOD mice are currently in progress (A. Herbelin, personal communication) required to clarify the implication of *i*Vα14 NKT cells in experimental diabetes.

Recent reports showed that the administration of α-GalCer alone or associated with IL-7 protects NOD mice from diabetes, even when it is started after the onset of insulitis [54, 63, 95]. The protection afforded by α-GalCer treatment was probably dependent on IL-10 and associated with the suppression of both T- and B-cell autoimmunity, as well as with a polarized Th2-like response in spleen and pancreas of NOD mice. The mechanisms implicated are not yet completely defined, but a recent study using a transgenic model, showed that *i*Vα14 NKT cells did not block the initial activation and expansion of effector diabetogenic T lymphocytes but prevented their differentiation into effector cells [92]. Taken together, these findings raise the possibility that α-GalCer treatment might be used to prevent the onset and recurrence of human autoimmune diabetes.

Experimental Allergic Encephalomyelitis (EAE)

Deficiencies in the NKT cell population have been observed in multiple sclerosis and mouse strains susceptible to experimental autoimmune encephalomyelitis (EAE) [93, 94]. Similar to experimental autoimmune diabetes, activation of *i*Vα14 NKT cells by α-GalCer protects susceptible mice against EAE [70, 95]. This treatment failed to protect CD1d$^{-/-}$, IL-4$^{-/-}$ and IL-10$^{-/-}$ strains, indicating the implication of these cytokines, as well as the requirement for an intact CD1d antigen-presenting pathway [95]. At the same time, another study showed that EAE could be prevented by the treatment with an analogue of α-GalCer with a truncated sphingosine chain. [70], which induced a preferential production of IL-4 by *i*Vα14 NKT cells. However, the effect of α-GalCer treatment in this model is complex, because in some cases it can increase the severity of the disease. Indeed, while simultaneous activation of *i*Vα14 NKT cells with α-GalCer and myelin-reactive T cells exacerbates EAE, prior α-GalCer treatment become protective [96]. This contrasting effect was explained by an enhanced Th1 response against myelin basic protein associated with IFN-γ production in the case of exacerbation or a Th2 response and IL-4 production in the case of protection [96].

Although activation of *i*Vα14 NKT cells by α-GalCer treatment was generally efficient in models of spontaneous disease in autoimmune-prone mice, results may be more complex in conditions where the pathology is induced by exogenous antigens, which is the case of experimental EAE. Taken together, these results have important implications for designing therapeutic strategies to polarize NKT cells toward a Th2 or Th1 profile.

iVα14 NKT cells and infection

*i*Vα14 NKT cells might play a critical role in a variety of infectious diseases, probably through their capacity to influence and amplify both innate and acquired immune responses. Some examples are shown in the following paragraphs.

Bacteria

A clear example of the influence of *i*Vα14 NKT cells in innate immune responses is the generalized Shwartzman reaction, which consists in a lethal shock syndrome caused by priming and challenge of mice with low doses of lipopolysaccharide (LPS) [97]. In this syndrome, a cascade of cytokines is produced following LPS injection, starting with IL-12, which is critical for induction of IFN-γ, which, in turn, will initiate TNF-α secretion, causing liver injury and death. It is noteworthy that *i*Vα14 NKT cell-deficient mice (CD1d$^{-/-}$ and Jα18$^{-/-}$) were resistant to LPS-elicited mortality. In these mice both IFN-γ and TNF-α production were reduced, while IL-12 levels remained similar to those observed in wild-type mice. A direct stimulation of *i*Vα14 NKT cells by LPS was not demonstrated, suggesting that the critical role of these cells in the generalized Shwartzman reaction is mediated by their IFN-γ secretion in response to IL-12 [97].

Granuloma formation followed by a T cell-mediated immune response is critical in preventing clinical disease following *Mycobacterium tuberculosis* infection. A major study using mice injected with deproteinized phosphatidylinositolmannoside-enriched cell walls prepared from *M. tuberculosis* clearly demonstrated the requirement of *i*Vα14 NKT cells in granuloma formation [98]. Treated mice develop a granuloma-like lesion, in which *i*Vα14 NKT cells are not only predominant, but crucial for the granulomatous response, because the latter does not occur in Jα18$^{-/-}$ mice. The early attraction of *i*Vα14 NKT cells to inflammatory lesions requires IP-10 [99]. Although CD1d-restricted *i*Vα14 NKT cells are not necessary for optimal immunity against *M. tuberculosis* [100], specific activation of these cells by α-GalCer treatment protects susceptible mice from tuberculosis by reducing the bacterial burden in the lungs, decreasing tissue injury and prolonging survival [101]. This protection is CD1d-dependet but further studies are required to better define the mechanisms implicated.

Protozoan parasites

Trypanosoma cruzi and *Plasmodium sp* are protozoan parasites which cause Chagas' disease or malaria, respectively, in humans. It has been proposed that self and protozoan-derived glycophosphoinositol (GPI)-anchored surface antigens of Plasmodium and Trypanosoma are natural ligands of CD1d and that the CD1d-NKT cell pathway regulates IgG responses to these antigens [102]. In agreement with this idea, other data indicate that in NKT-cell-deficient (Jα18$^{-/-}$ and CD1d$^{-/-}$) mice chronically infected with *T. cruzi* the antibody response to one GPI-modified protein was decreased [103]. Nonetheless, these results are controversial, because it was demonstrated that MHC class II molecules, rather than CD1d, are crucial for anti-circumsporozoite IgG responses during immunization with irradiated plasmodium parasites [104]. In addition, even though GPI-anchored mucin-like glycoproteins (GPI mucins), glycoinositolphospholipids (GIPLs), and their phosphatidylinositol moieties bound to rCD1d and inhibited the stimulation of a NKT hybridoma by the α-GalCer-CD1 complex, these GPI anchors and related structures were unable to activate NKT cells *in vitro* or *in vivo* [105].

These studies indicate that dominant CD1d-restricted immune responses are not elicited by GPI mucins and related structures expressed by these parasites, suggesting that other mechanisms such as the presence of pro-inflammatory cytokines (IL-12, IL-18), can induce the activation of iVα14 NKT cells [66] during these infections. In mice infected with *P. yoelli,* CD4^{+} NKT cell frequencies were decreased in the liver, but accompanied by a corresponding increase in the DN NKT subset, which directly inhibited pathogen growth in hepatocytes *in vitro via* an IFN-γ-dependent mechanism [106]. It is therefore possible that IL-12 and IL-18 produced during infection initiate the secretion of IFN-γ by *i*Vα14 NKT cells and influence the outcome of the disease. This might also explain the greater parasitemia observed in both *P. yoelli* or *T. cruzi* infected NKT-cell-deficient Jα18$^{-/-}$ or CD1d$^{-/-}$ mice [103, 107].

In addition to the possible physiological role of NKT cells in the control of protozoan infections, it has been reported that a specific *in vivo* activation of *i*Vα14 NKT cells with α-GalCer results in a strong anti-malarial activity. This effect is mediated through IFN-γ-secreting NKT cells, in both *P. yoelii*- and *P. berghei*-infected mice [108]. Interestingly, co-administration of α-GalCer and suboptimal doses of irradiated sporozoites or recombinant viruses

expressing a malaria antigen greatly enhances the level of protective anti-malarial immunity in mice [107]. This adjuvant effects of α-GalCer, which require CD1d molecules, *i*Vα14 NKT cells, and IFN-γ, open new perspectives for the design of more effective vaccines against malaria and other intracellular pathogens.

Virus

Hepatitis B virus (HBV) transgenic mice treated with a single injection of α-GalCer abolished HBV replication [109]. The antiviral effect of α-GalCer was inhibited in mice genetically deficient for either IFN-γ or the IFN-α/β receptor, showing the implication of these cytokines. α-GalCer treatment also protected mice against the cytopathic, diabetogenic encephalomyocarditis virus (EMCV-D) [110]. It is surprising that in this model, where α-GalCer treatment is effective, the *i*Vα14 NKT cells seem to play no major role in the natural immune response against these infections since $CD1d^{-/-}$ but not $J\alpha18^{-/-}$ mice were more susceptible to EMCV-D than wild type animals [110]. Similarly, T cells, which are CD1d-restricted but non-reactive to α-GalCer, are activated in response to hepatocytes expressing hepatitis B viral antigens in a transgenic mouse model of acute HBV infection [29]. Taken together these data show that *i*Vα14 NKT cells activated by α-GalCer may amplify protective antiviral responses. However, during the physiological development of EMCV-D or HBV infections, CD1d-dependent cells distinct from *i*Vα14 cells are required, even though it remains possible that the effect of *i*Vα14 cells could not be demonstrated because transgenic mice were used in the HBV model.

Recently, impaired clearance of herpes simplex virus and viral Ags, and more florid acute infection in both $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice was reported [111], showing the physiological influence of *i*Vα14 NKT cells in this experimental model. $CD1d^{-/-}$ mice also presented alterations in illness, viral clearance, and IFN-γ production in an experimental model of respiratory syncytial virus (RSV) [112]. *i*Vα14 NKT cell-deficient $J\alpha18^{-/-}$ mice were not tested in this model of RSV, but activation of *i*Vα14 NKT cells by α-GalCer resulted in reduced illness and delayed viral clearance [112].

Little is known about the implication of invariant NKT cells in human viral diseases, but recent studies reported that *i*Vα24 cells are potential targets for human immunodeficiency virus (HIV)-1 infection. Indeed, both resting and activated human $CD4^+$ $i\text{V}\alpha24^+$ T cells express high levels of the HIV-1 coreceptors CCR5 and Bonzo (CXCR6), and were more susceptible to infection then conventional T cells [113-115]. The possible implication of $i\text{V}\alpha24^+$ T cells in HIV infection was sustained by results showing that they were markedly and selectively depleted in the peripheral blood of HIV-1-infected individuals [116]. In contrast with the protective role ascribed to NKT cells in murine models of viral infection, these findings suggest that $CD4^+$ NKT cells may have a potential role during HIV-1 transmission and spread *in vivo*. These data open the prospect of employing $V\alpha24^+$ T cells as targets to new therapeutic anti-HIV approaches.

Tumor rejection

The first demonstration that $i\text{V}\alpha14^+$ NKT cells can be critical for anti-tumor immune responses came from studies by Taniguchi's group showing that these cells are essential mediators in IL-12-induced rejection of tumors in a model of melanoma [117]. The glycolipid α-GalCer, first discovered by its anti-tumor properties, exerts its beneficial effects through its action on $i\text{V}\alpha14^+$ NKT cells [21, 118]. Thus, the anti-tumor effects of α-GalCer against various tumor cells of different origins including melanomas, lung, colon, and renal cell carcinomas, erythroleukemias and other hematopoietic malignancies depends on the activation of $i\text{V}\alpha14^+$ NKT cells [58, 71, 118, 119].

Recently, some important studies have further delineated the sequence of major events that result in the anti-metastatic activity of α-GalCer. Early activated $i\text{V}\alpha14^+$ NKT cells promptly produce IFN-γ, which will, in turn, in association with IL-12 produced by dendritic cells, promote a massive production of IFN-γ by classical NK cells [58, 120, 121]. The anti-metastatic activity of α-GalCer required the sequential production of IFN-γ by both NKT cells and NK cells to become effective [58]. These data come from a series of elegantly performed NKT cell and/or NK cell adoptive transfer experiments, with donor/recipient combinations using $J\alpha18^{-/-}$, $CD1d^{-/-}$, $IFN\text{-}\gamma^{-/-}$ or $RAG^{-/-}$ strains [58]. The anti-metastatic activity of α-GalCer is primarily dependent on the IFN-γ produced by $i\text{V}\alpha14^+$ NKT cells, but does probably not require cytotoxic activity because the anti-metastatic effect was not abrogated in FasL-, perforin-, TNF- or TRAIL-deficient mice [58]. In fact, it was recently demonstrated that IFN-γ produced by *i*Vα14 or NK cells is necessary to inhibit tumor angiogenesis [122], probably by decreasing the proliferation of tumor-associated endothelium, thereby indirectly restraining tumor neo-vascularization and inducing tumor hypoxia.

Although *i*Vα14 NKT activated exogeneously by α-GalCer or IL-12 display a clear anti-tumor activity, little is known about their possible physiological role in tumor immuno-surveillance. Recent reports demonstrated a critical role for *i*Vα14 NKT cells in immuno-surveillance of methylcholanthrene (MCA)-induced fibrosarcomas, in the absence of exogenous stimulatory factors, using $J\alpha18^{-/-}$ mice [123]. It will be of great interest to determine the mechanism by which *i*Vα14 NKT cells may contribute to the control of tumor development and whether spontaneous tumors will arise more frequently in NKT-cell-deficient mice.

CONCLUSIONS

Clearly, the influence of *i*Vα14 NKT cells on immune responses can range from protective to aggressive. Many studies support the concept that the molecular interactions between *i*Vα14 NKT cells and APCs, the nature of the stimulus and the cytokines present in their environment, dictate what will be the impact of these cells on autoimmunity, host defense and antitumor immunity. Depending on the nature of the CD1d/glycolipid/TCR interaction, the balance of co-stimulatory factors (CD4, NK receptors, CD40L or CD28), the type of APC or cytokines (IL-7, IL-12 or IL-18) in the microenvironment, *i*Vα14 NKT cells can amplify or inhibit divers immune responses. It is therefore essential to define the major parameters, which control the functional properties of *i*Vα14 NKT cells (Th1 versus Th2 profile), to establish

efficient and secure therapeutic protocols using α-GalCer or other new treatments affecting NKT cells.

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