REVIEW

Arch. Immunol. Ther. Exp., 2009, **57**, 117–128
PL ISSN 0004-069X
DOI 10.1007/s00005-009-0009-x

# NKT cells: from totipotency to regenerative medicine

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**Received:** 2008.11.12, **Accepted:** 2009.01.05, **Published online:** 2009.03.31



## Abstract

The recent discovery that natural killer T (NKT) cell nuclei are totipotent opens a novel avenue for further understanding NKT cell function in normal and diseased states. The progeny of a cloned mouse harboring the in-frame rearranged Vα14--Jα18 T cell receptor in one allele showed a significant increase in NKT cell number compared with wild-type or littermate control mice that possessed a different TCR. Importantly, NKT cells from such progeny produced both interferon-γ and interleukin-4, a hallmark of NKT cells. In these progeny, NKT cell development appeared to be instructively, rather than permissively, determined. Using embryonic stem cells prepared via the somatic cell nuclear transfer of NKT nuclei, relatively mature NKT cells were induced under conditions permissible for T cell induction. Furthermore, these NKT cells matured autonomously upon injection into mice, resulting in an antigen-specific adjuvant effect.

**Key words:** NKT cell, totipotency, embryonic stem cell, instructive, permissive, differentiation.

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## INTRODUCTION

Natural killer T (NKT) cells are characterized by their unique expression of both the T cell receptor (TCR) and natural killer (NK) receptors on the cell surface. Because NK receptor expression in activated $CD8^+$ T cells is dependent upon developmental stage, activation state, and genetic background, it is difficult to define NKT cell types precisely. Although TCRs expressed on NKT cells may consist of several different V-J combinations, the most studied and well-characterized subset harbors the invariant Vα14-Jα18 in mouse and Vα24-Jα18 in human (hereafter referred to as *i*NKT cells). Whereas the development of conventional T cells is restricted by polymorphic major histocompatibility complex (MHC) class I and class II molecules, the development of *i*NKT cells is contingent upon CD1d, a MHC class I-related antigen-presenting glycoprotein that is monomorphic (Bendelac et al. 2007; Kronenberg 2005; Taniguchi et al. 2003). Invariant NKT cells are self-reactive and behave similarly to regulatory T cells in that they secrete various types of cytokines and thus may be important in the immune system. Indeed, accumulating evidence demonstrates that *i*NKT cells play pleiotropic roles in many of the disease models studied so far (reviewed in Bendelac et al. 2007; Tupin et al. 2007). Therefore, *i*NKT cells may represent a potential target for therapeutic intervention in a variety of autoimmune diseases, tumor growth, and bacterial and viral infections (Kronenberg and Rudensky 2005; Tupin et al. 2007). In the first part of this review we describe the totipotent effect of NKT nuclei upon transfer into enucleated oocytes. In the second part we discuss the mode of NKT cell differentiation. Finally we will discuss the generation of *i*NKT cells from embryonic stem cells (ESCs) and their potential therapeutic use in regenerative medicine.

## TOTIPOTENCY OF THE *i*NKT CELL NUCLEUS

It was not until 1996 and the successful birth of a lamb cloned via somatic cell nuclear transfer (SCNT) that the scientific world accepted the notion of cloning a mammal (Campbell et al. 1996). In 1997, Dolly was cloned from a mammary gland-derived cell line, demonstrating that cloning is possible even from a differentiated cell (Wilmut et al. 1997). There is, however, a caveat to such an immediate conclusion. Totipotency (i.e. the potential to reset genomic programming and give rise to a cloned animal upon SCNT) is thought to be imparted to only a limited set of cells, such as fertilized eggs (zygotes), first-cleavage blastomeres, and perhaps somatic stem cells; therefore, it is impossible to preclude the po-

ssibility that Dolly originated from a stem cell present in cultured cells. The controversy as to whether terminally differentiated cells induce totipotency upon direct nuclear transfer is due, in part, to the lack of genetic trait(s) through which to ascertain the origin of cells giving rise to cloned animals. In the case of Dolly, DNA microsatellite analysis demonstrated the authenticity of the clone, but it remains unclear whether the clone originated from a differentiated cell or a stem cell (Ashworth et al. 1998). It is thus desirable to use cells harboring identifiable genetic trait(s) to demonstrate the totipotency of differentiated cells. Since T and B cells circulating in the peripheral tissues harbor rearranged TCRs/B cell receptors (BCRs) upon emigration from the thymus or bone marrow, respectively, these cells can be considered fully differentiated. Antigen-specific TCRs/BCRs are produced via recombination of the V and J genomic DNA fragments, and these changes in genomic configuration are detectable via Southern blot analysis, making it possible to ascertain the origin of the cell that gave rise to the cloned individual. However, a previous report that peripheral T and B cells cannot be used to generate a cloned animal via direct nuclear transfer suggests that differentiated cells are not totipotent. Indeed, two-step nuclear transfer involving ESCs and tetraploid complementation was required to generate cloned mice (Hochedlinger and Jaenisch 2002).

Similarly, cloned mice can be generated from post--mitotic neuronal cells via two-step nuclear transfer (Eggan et al. 2004; Li et al. 2004). Although these mice stem from "terminally differentiated cells", the fact that a two-step procedure is required indicates that, similar to T and B cells, post-mitotic neuronal cells are not totipotent. From these data it appears that these differentiated cell types are able to give rise to the fetus but not to the placenta; that is, they are not truly totipotent. However, it remains unclear whether terminally differentiated cells exhibit totipotency upon SCNT. In this regard, our finding that NKT cells can be used to generate cloned mice and placenta upon SCNT without resorting to a two-step procedure demonstrates that terminally differentiated cells are capable of producing a cloned animal via genomic reprogramming (Fig. 1).

Since all cells in the cloned mouse and placenta are derived from the NKT cell nucleus, all must possess the rearranged *Vα-Jα* specific to NKT cells (Vα14--Jα18) at least on one allele; consequently, the origin of the clone is readily identifiable (Inoue et al. 2005). Intriguingly, the efficiency of generating ESCs upon SCNT is much higher with NKT cells than with T or B cells (Hochedlinger and Jaenisch 2002; Inoue et al. 2005). It is noteworthy that the efficiency of establishing ESCs from T or B cells is quite low compared with that using other cell types (Hochedlinger and Jaenisch 2002). Moreover, it is remarkable that mice cloned from NKT cells show no apparent abnormality after birth, grow normally, and are fertile (Inoue et al. 2005; Wakao et al. 2007; Wakao et al. unpublished observation); by contrast, findings reported to date show some defect in conceptus or as neonates, such as large offspring syndrome, prolonged gestation, dystocia (strenuous labor), fetal and placental edema, hydrallantois and hydramnios, abnormal organ size, respiratory problems, or perinatal death (Cibelli et al. 2002; Farin et al. 2006; Hochedlinger and Jaenisch 2003; Jaenisch and Young 2008; Yang et al. 2007; Young et al. 1998). Notably, most of these defects appear to be secondary to defects in placental function (Constant et al. 2006). This lack of defect among mice cloned from NKT cells may reflect a unique feature of the NKT cell nucleus that facilitates genomic reprogramming. Given that reprogramming of genomic imprinting, histone modification, and X-chromosome inactivation plays a pivotal role in the induction of totipotency upon nuclear transfer, it would be interesting to elucidate the underlying molecular mechanisms that impart such a unique ability. Such knowledge would provide a novel avenue for the use of NKT cell nuclei in regenerative medicine.

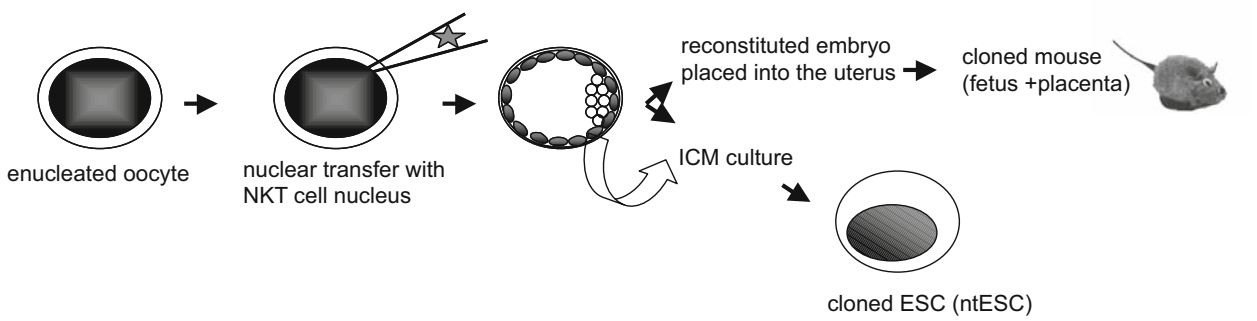


**Fig. 1.** Generation of a cloned mouse from an NKT cell nucleus and cloned embryonic stem cells (ntESCs) via SCNT. The nucleus from an *i*NKT cell is transferred into an enucleated oocyte. The reconstituted embryo is then placed into the uterus of a pseudopregnant mouse. The conceptus develops into placenta and fetus, giving rise to a cloned mouse. At the blastocyst stage of the reconstituted embryo, cloned ESCs (ntESCs) can be derived from the inner cell mass (ICM).

## A NOVEL MOUSE MODEL TO STUDY THE MODE OF *i*NKT CELL DEVELOPMENT

*Misconceptions regarding the instructive versus permissive mode in lymphocyte development*

The fact that *i*NKT cell development is contingent upon CD1d, but not upon MHC class I or class II, has prompted many researchers to question the origin of *i*NKT cells and their mode of development. Despite extensive investigation, however, it has not been possible to identify the progenitor(s) of *i*NKT cells with certainty. However, a recent genetic study has strongly suggested that they stem from $CD4^+CD8^+$ double-positive (DP) thymocytes (Egawa et al. 2005). Whether *i*NKT cell development is predetermined before the DP stage is beyond the scope of this review. Those who are interested in such a debate may refer to recent reviews on the topic (Bendelac et al. 2007; Godfrey and Berzins 2007; Kronenberg and Gapin 2002). In this section we will discuss a long-unresolved issue: whether extrinsic signaling "permissively" or "instructively" determines the fate of *i*NKT progenitor cells.

Cell lineage commitment in a multipotent stem cell is governed by combinatorial interactions among extrinsic and intrinsic factors. Extrinsic factors influence fate determination in multipotent progenitor cells. In such circumstances, cell lineage commitment is determined in a permissive or instructive manner. In the permissive model, the decision to differentiate into a particular lineage is made independently of extrinsic factors; instead, the *raison d'être* of these factors is to support the survival and proliferation of committed cells (Fig. 2A, right panel). In the instructive model, extrinsic factors drive progenitor cells to select one lineage at the expense of others (Fig. 2A, left panel) (Morrison et al. 1997).

Intensive investigation in neural crest stem cells has demonstrated that neural cell fate is determined instructively by cytokines such as bone morphogenetic protein-2 (BMP-2), glial growth factor (GGF), and transforming growth factor (TGF)-β (Shah et al. 1994; Shah et al. 1996). BMP-2 promotes differentiation into autonomic neurons, whereas GGF and TGF-β induce Schwann (glial) cells and smooth muscle cells, respectively. Similarly, ciliary neurotrophic factor cues CNS stem cells to form astrocytes (Johe et al. 1996).

In hematopoiesis, however, the relative contribution of these two mechanisms remains in debate (Mayani et al. 1993; Metcalf 1991). Virus-mediated erythropoietin receptor (EPOR) transduction into bone marrow cells induced the formation of erythrocytes, granulocytes, macrophages, and megakaryocytes in the presence of EPO, indicating that EPO possesses no instructive potential in dictating erythrocyte lineage commitment (Dubart et al. 1994). EPOR ablation resulted in a marked decrease in erythrocytes, although the generation of erythrocyte progenitors remained relatively stable (Wu et al. 1995). This type of combined gain- and loss-of-function study demonstrates that EPO/EPOR-mediated signaling plays a permissive role in erythropoiesis. Similarly, the transgenic expression of granulocyte colony-stimulating factor (GCSF) receptor results in the development of primitive multipotent progenitors, but not exclusive commitment to the myeloid lineage, again reinforcing the notion that GCSF-elicited signaling does not operate instructively in determining the fate of such progenitor cells (Yang et al. 1998). Thus far, most if not all studies on cytokine/cytokine receptor signaling support the permissive model. Moreover, one must be cautious when interpreting data showing that constitutive activation or complete ablation of a given signal results in the generation of lineage A over lineage B, as demonstrated below.

Notch1 plays a crucial role in T lineage commitment, based on an experiment in which Notch1 over-expression triggered T cell production in bone marrow, whereas few B cells were produced (Pui et al. 1999). Furthermore, the conditional deletion of Notch1 in hematopoietic lineages revealed that B cells differentiated in the thymus, whereas few T cells developed (Radtke et al. 1999). These results indicate that Notch may function as more than a growth factor. However, it remains unclear whether Notch determines the fate of progenitor cells *per se*, or whether it merely supports the survival and proliferation of T cell lineage-committed progenitors.

Accumulating evidence shows that antigen receptor-mediated signals are required for lymphocyte development. Mice lacking the TCRVα chain exhibit developmental arrest at the DP stage (Mombaerts et al. 1992). However, it is unknown whether the formation of a TCR complex instructively or permissively governs lymphocyte development. It has been proposed that the formation of a pre-TCR complex composed of the TCRVβ chain together with pTα and CD3ε instructively induces αβT cell lineage commitment (Aifantis et al. 1998). Another report suggested that αβ/γδT cell lineage diversification occurs well before TCR rearrangement, thus suggesting permissive commitment (Kang et al. 2001). Recent studies have suggested that commitment to αβ T cells versus γδ T cells is contingent on the strength of the γδTCR signal (Haks et al. 2005; Hayes et al. 2005). Nevertheless, these models raise questions as to whether such lineage commitment is instructively or permissively determined.

It has also been long disputed whether the decision to become a helper or cytotoxic T cell is instructive or permissive. In this case, the CD4 or CD8 molecule functions as a co-receptor to monitor whether the preformed TCR complex can interact with MHC class II or I. The transgenic expression of CD8 substantially improves MHC class I-specific CD8 selection while having little effect on class II-specific CD4 selection, suggesting that $CD8^+$cell lineage commitment is instructively determined (Robey et al. 1991). Subsequent studies using MHC class I knockout mice confirmed the absence of $CD8^+$ cells. However, the reintroduction of transgenic CD4 into these mice restored the $CD8^+$ cell population and *vice versa* (Davis et al. 1993; Robey et al. 1994). These studies indicate that the decision to form $CD4^+$ or $CD8^+$

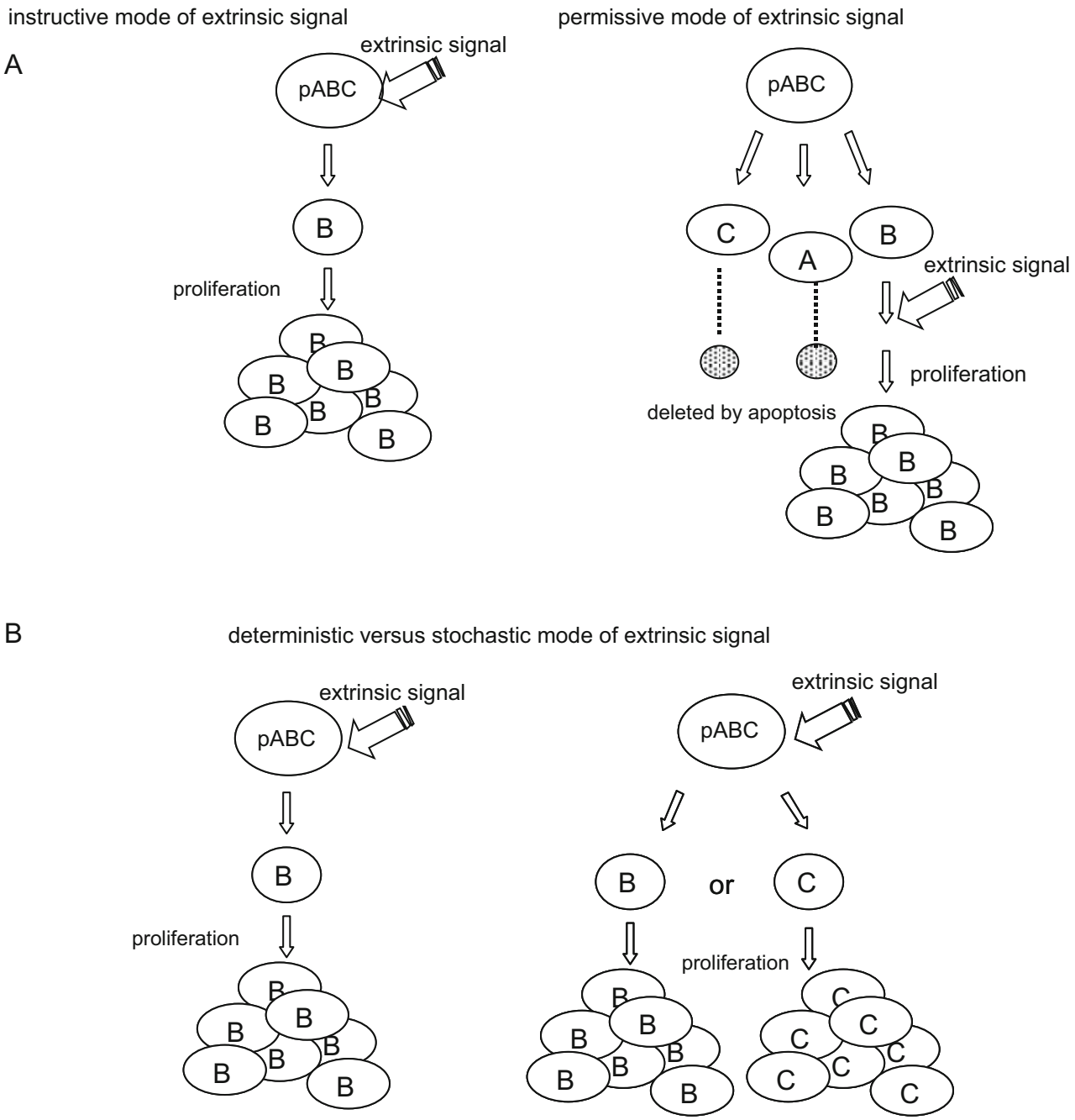


**Fig. 2. A:** Instructive versus permissive mode of extrinsic signaling. In the instructive mode, the extrinsic signal constrains the fate of the multipotent progenitor cell pABC to one lineage (e.g. lineage B at the expense of lineages A and C). In the permissive mode, the multipotent progenitor cell pABC simultaneously gives rise to lineages A, B, and C, independently of the extrinsic signal. However, the extrinsic signal supports the survival/proliferation of the selected lineage alone (in this case, lineage B). Therefore, lineages A and C cannot survive and are eliminated through apoptosis. **B:** Deterministic versus stochastic mode of instructive signaling. In the deterministic mode, the extrinsic signal constrains the fate of the multipotent progenitor cell pABC to lineage B alone with 100% probability. In this case, lineages A and C are not generated (instructive extrinsic signaling). In the stochastic mode, the extrinsic signal constrains the fate of the multipotent progenitor cell pABC to lineage A or B at a given probability. Note that when lineage A arises through extrinsic signaling, lineages B and C are not generated. When B lineage is induced via the extrinsic signal, lineages A and C are not generated (instructive extrinsic signaling).

cells is not dictated by CD4 or CD8 *per se*, but rather by CD4/8-mediated signaling that contributes to the survival or proliferation of the committed cells. In the recently proposed kinetic signaling model, the transient reduction of CD8 DP thymocytes is a proposed prerequisite for cell fate determination in $CD4^+$ or $CD8^+$ T cells (Bosselut et al. 2003). Accordingly, persistent TCR signaling in intermediate cells directs them to become $CD4^+$ cells, whereas the cessation of TCR signaling results in co-receptor reversal and generates $CD8^+$ cells. However, it is unclear whether $CD4^+$ versus $CD8^+$ lineage is instructively or permissively determined. The re-

cent discovery of Th-POK/cKrox as a transcription factor responsible for CD4 lineage determination is an important clue to the above issue (He et al. 2005; Sun et al. 2005). Since the retrovirus-mediated expression of Th-POK in hematopoietic cells, or its T cell-specific transgenic expression, not only reinstates normal development of class II-restricted T cells to the CD4 lineage in $HD^{-/-}$ mice in which no $CD4^+$ cells is present, but also engenders redirection of class I-restricted cells to the CD4 lineage in both $HD^{-/-}$ and wild-type background (He et al. 2005), and the absence of functional Th-POK is sufficient to drive development of the CD8 lineage, the identification of the extrinsic stimuli that induce the expression of *Th-POK* will help to decipher the mode of CD4 versus CD8 lineage commitment.

The above uncertainty is largely due to a problem inherent in the experimental design: the lack of an appropriate read-out system. If cells are analyzed only during late events (i.e. after proliferation), it is impossible to determine whether such cells represent the progeny of instructively or permissively fated cells. Therefore it is imperative to examine early events in lymphocyte development, during which time pre-committed and committed cells coexist.

Great effort has been made to understand the development of *i*NKT cells, and it has been suggested that their commitment is "selected" during thymocyte development (Gapin et al. 2001). According to this hypothesis, cells undergo random TCRα rearrangement. Those expressing Vα14-Jα18 diverge from $CD4^+CD8^+$ DP cells and become *i*NKT cells. The fact that few *i*NKT cells are present in *CD1d* knockout mice suggests that these cells are only detectable after vigorous post-selection proliferation. Therefore it is probable that such cells represent the progeny of cells committed to be *i*NKT cells after extensive proliferation. If this is the case, it is impossible to determine whether *i*NKT cell commitment is instructively or permissively determined. Moreover, given that the probability of forming Vα14-Jα18 is extremely low (<1 in 50,000) due to random TCRα rearrangement and N-nucleotide insertion between the V and J segments, it is impossible to determine whether the resultant cells represent pre-committed progeny following the normal course of development or the descendants of pre-committed conventional T cells undergoing further rearrangement to alter cell fate or induce apoptosis (Bendelac et al. 1997; Gapin et al. 2001). It is noteworthy that this "selection model" does not appear to be instructive in nature. Similarly, a study using transgenic Vα14-Jα18 demonstrated that both the preponderance of *i*NKT cells in thymocytes and a biased decrease in $V\beta7^+$ and $V\beta8^+$TCR in $CD8^+$ cells favors the selection of Vα14-Jα18 (Bendelac et al. 1996; Benlagha et al. 2000). Nonetheless, it is unclear whether the predominance of *i*NKT cells in thymocytes is the result of instructive commitment or permissive selection. Moreover, such bias in TCRβ does not necessarily ensure *i*NKT lineage commitment upon the assemblage of Vα14-Jα18 and TCRβ7 or TCRβ8; it is possible that such a combination may instead inhibit positive selection imposed by $V\beta8^+/CD8^+$ cells.

As discussed in the case of EPO, GCSF, αβ versus γδ T cells, and $CD4^+$ versus $CD8^+$ lineage commitment, use of the Vα14-Jα18 transgene in experiments creates uncertainty in that the spatiotemporal expression of Vα14-Jα18 is not ensured, and cells are not allowed to delete such a transgene through further rearrangement. It is thus desirable to create a novel mouse model in which the expression of Vα14-Jα18 TCR is under the control of endogenous promoter(s) and enhancers that promote proper expression and remain subject to further rearrangement. Such a model would provide important insight into the nature of *i*NKT commitment.

*Generation of a novel mouse model*

In a previous study we generated fertile cloned mice from NKT cells using SCNT (Inoue et al. 2005). After crossing with C57BL/6 females, we obtained progeny harboring the in-frame rearranged V-J specific to Vα14-Jα18, the in-frame rearranged Vβ8S2-Dβ1-Jβ2S5 specific to TCRβ8.2, and progeny harboring both rearrangements (Fig. 3A). Among these progeny, we focused on those harboring the in-frame rearranged Vα14-Jα18 specific to the *i*NKT cell TCR in one allele (hereafter referred to as Vα14-Jα18 mice, Fig. 3A-(2)). This genomic configuration allows the further deletion of Vα14-Jα18 via recombination and the transcription of another *Tcr*α from the other allele, which may be useful in studying the mode of *i*NKT cell development. Indeed, Vα14-Jα18 mice show normal γδ T cell numbers and the ratio of αβ to γδ T cells is almost identical to that in the wild-type (Wakao et al. 2007), implying *Tcr*δ transcription from the other allele. Intriguingly, the number of *i*NKT cells increased markedly in the Vα14-Jα18 mice, which is consistent with observations in Vα14-Jα18 transgenic mice (Bendelac et al. 1996). Analyses of cell surface molecules expressed on *i*NKT cells revealed that these cells were mostly $CD4^+CD8^-$ or $CD4^-CD8^-$, whereas $CD4^-CD8^+$ cells were present in secondary organs such as the liver. However, the expression profile of NK receptors, such as Ly49A and Ly49D, and of CD25 in Vα14-Jα18 mice was equivalent to that found in control mice in the thymus and spleen, in which the percentage of $NK1.1^+$ cells decreased concomitantly with an increase in $CD44^{dull}$ cells. In contrast, the $CD24^+$ cell population increased in thymocytes from Vα14-Jα18 mice. This discrepancy indicates that *i*NKT cells in Vα14-Jα18 mice are composed of at least two different populations, including pre-committed (CD1d-nonreacted) and post-committed (CD1d-reacted) cells.

*The iNKT cell population is composed of CD1d-reacted and -nonreacted cells*

Previous studies have reported that *i*NKT cells are present in mice even in the absence of *CD1d* (Hong et al. 2001; Singh et al. 2001), although such cells are very

scarce and their nature remains unclear. This observation is somewhat strange given that the development of *i*NKT cells is dependent on CD1d. Nonetheless, it is possible that these cells represent an immature *i*NKT population expressing Vα14-Jα18 and recombination-activating genes at the DP stage. Because the presence of both pre-committed (CD1d-nonreacted) and post-committed (CD1d-reacted) cells is necessary to study the role of extrinsic signaling in progenitor cell fate, these *i*NKT cells in CD1d$^{-/-}$ mice most likely represent a pre-committed population. The presence of pre-committed *i*NKT cells becomes more apparent when the allele harboring Vα14-Jα18 is expressed in *CD1d*$^{-/-}$ mice. In these mice, *i*NKT cells represented ~1% of thymocytes, and staining for *i*NKT cells with various monoclonal antibodies revealed that although there were few CD69$^{+}$, CD44$^{high}$, or NK1.1$^{+}$ cells, the expression of CD24 (an immature marker) was elevated relative to the control (Fig. 4A) (Wakao et al. 2007). These cells were also present in the thymocytes of Vα14-Jα18 mice. Thus the *i*NKT population in Vα14-Jα18 mice is composed of both pre-committed (CD1d-non-reacted) and post-committed (CD1d-reacted) cells (Fig. 4A). Importantly, *i*NKT cells in Vα14-Jα18 mice produced both interferon (IFN)-γ and interleukin (IL)-4 upon appropriate stimuli, whereas Vα14-Jα18 transgenic mice failed to produce IFN-γ upon TCR engagement (Bendelac et al. 1996; Wakao et al. 2007). Therefore, Vα14-Jα18 mice are ideal for examining the mode of *i*NKT cell development.

Ignoring the precise definition of permissive versus instructive commitment of progenitor cells has led to a great deal of confusion regarding these terms. In the case of *i*NKT cell development, if extrinsic signals mediated through interaction with CD1d operate in an instructive manner, progenitor cells are fated to become *i*NKT cells at the expense of other cell types (i.e. other cell types are not produced; Fig. 4B, left panel). In contrast, should such a signal operate in a permissive manner, progenitor cells should simultaneously give rise to many types of differentiated cells, including cytotoxic T (Tc) cells, helper T (Th) cells, and *i*NKT cells. In this case it is important to remember that CD1d-mediated signaling does not constrain the fate of progenitors *per se*, but it supports the survival or proliferation of the committed *i*NKT cells alone. Consequently, nascent Tc and Th cells are eliminated via apoptosis or forced to change their TCR to Vα14-Jα18 (Fig. 4B, right panel).

*Stochastic versus deterministic mode of extrinsic signaling*

The terms stochastic and deterministic may be employed in an instructive or permissive manner when discussing extrinsic signaling. In other words, an instructive signal can result in both stochastic and deterministic output, and the same is true for a permissive signal. When an extrinsic signal cues the progenitor cell pABC to commit to the B lineage with 100% probability, such a signal is said to operate deterministically in pABC cell fate (Fig. 2B, left panel). In contrast, if the extrinsic signal merely constrains pABC to adopt the B or C lineage at a given probability, such a signal is said to operate stochastically (Fig. 2B, right panel). This principle can also be applied to permissive signals and may thus support the survival/proliferation of permissively selected cell lineages whose destiny is determined either deterministically or stochastically (see above discussion of EPOR or GCSF receptor in transgenic mice). Given that a permissive signal selects a cell lineage to be rescued from cell death, an extrinsic signal may operate selectively. In this context, the word selective is equivalent to the word permissive. However, an antagonistic conception of permissive/selective is instructive, but not stochastic or deterministic, as described above (Wakao et al. 2007).

Evidence exists that *i*NKT cell development is instructively governed by CD1d-(Vα14-Jα18TCR) signaling. The first piece of evidence is the predominance of *i*NKT cells in Vα14-Jα18 mouse thymus. The second indication is CD1d-dependent association of Vα14-Jα18 with Vβ8; while 80% of TCRβ8$^{+}$/TCRβ$^{+}$ cells consist of *i*NKT cells in Vα14-Jα18 mouse thymocytes, only 20% of the population in Vα14-Jα18/*CD1d*$^{-/-}$ are *i*NKT cells. Third, immature *i*NKT cells in the DP stage are less likely to perform further rearrangement relative to immature conventional T cells, as evidenced by decreased *Rag* transcription (Wakao et al. 2007). Fourth, the lack of massive apoptosis and of significant number of fast-cycling cells in Vα14-Jα18 mouse thymocytes. These observations indicate that immature *i*NKT cells do obey their fate to be *i*NKT cells upon CD1d-(Vα14-Jα18TCR) signaling without striving to change the TCRα by operating the recombination machinery, and without dying massively by apoptosis.

In summary, the above data strongly support the hypothesis that extrinsic CD1d-mediated signaling instructively determines the fate of progenitor cells via Vα14--Jα18 TCR, resulting in the formation of *i*NKT cells at the cost of other cell types (Fig. 4B).

## REGENERATIVE MEDICINE USING *i*NKT CELLS

*Status quo of the iNKT cell study*

Invariant NKT cells play multiple roles in the immune system. In mice they maintain self tolerance and perform tumor surveillance (Godfrey and Kronenberg 2004; Kronenberg and Rudensky 2005). Although *i*NKT cells could conceivably be used to alleviate autoimmune diseases and graft-versus-host disease, as well as to induce tolerance in allogeneic cardiac transplantation, there are several hurdles to clinical application. The first problem is the Janus-like behavior of *i*NKT cells in immune regulation; that is, *i*NKT cells are both immunosuppressive and immunostimulatory in nature. Therefore it is necessary to elucidate the underlying molecular mechanisms of these opposing effects before clinical appli-

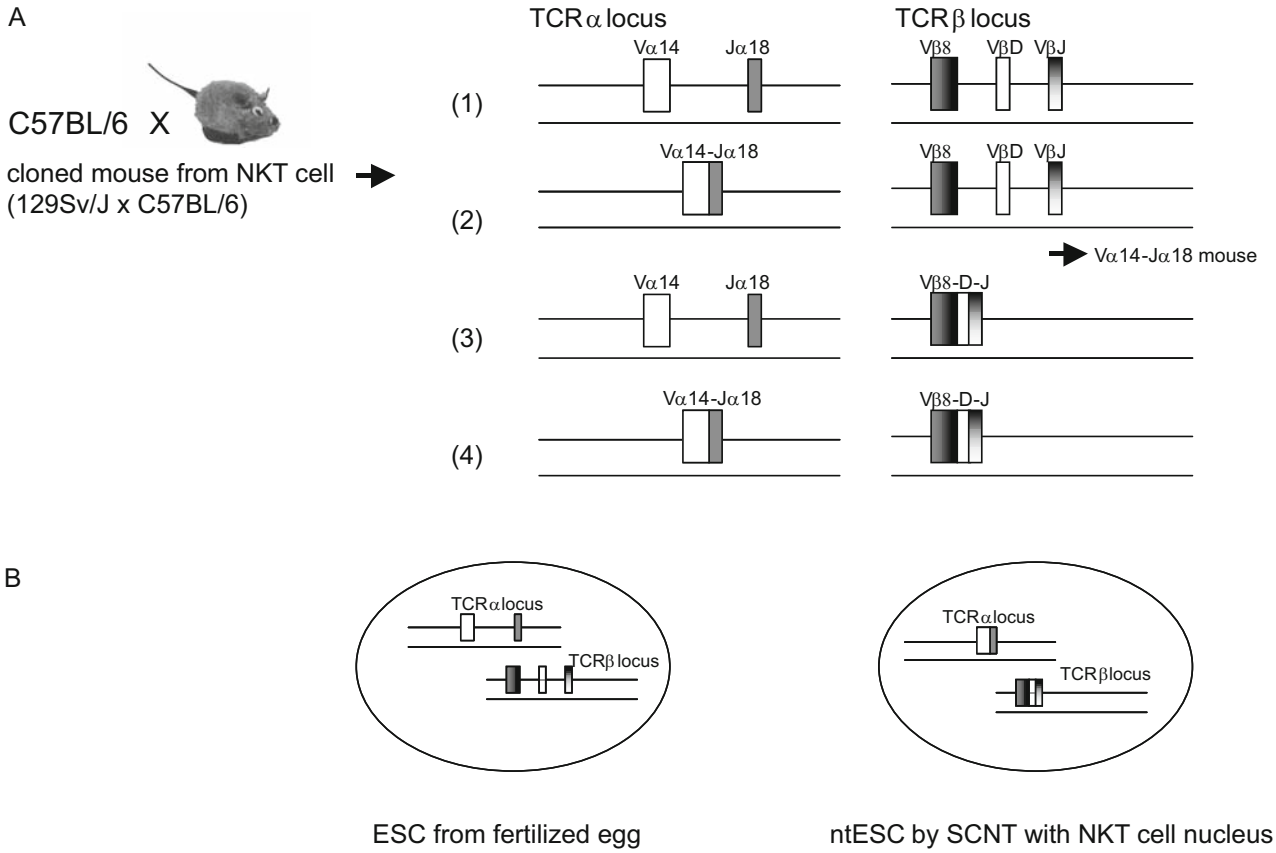


**Fig. 3. A:** Genomic configuration of the progeny of a cloned mouse crossed with a C57BL/6 female. Possible combinations of the *TCR*α and *TCR*β loci in the genome of the progeny. Only one allele is used to depict the presence of Vα14, Jα18, Vβ8, VβD, and VβJ. In-frame rearranged Vα14-Jα18 and Vβ8-VβD-VβJ are indicated by unified boxes. This genomic configuration is passed to the next generation. **B:** Genomic configuration of ESCs and ntESCs. ESCs prepared from a fertilized egg possess the non-rearranged Vα14 and Jα18 locus and Vβ8, VβD, and VβJ. In contrast, ntESCs prepared via SCNT using NKT nuclei harbor in-frame rearranged Vα14-Jα18 and Vβ8-VβD-VβJ in at least one allele.

cation is possible. The ability of *i*NKT cells to produce both Th1 cytokines, such as IFN-γ, and Th2 cytokines, such as IL-4, IL-10, and IL-13, undoubtedly plays a critical role in the immunoregulatory function of *i*NKT cells. The second piece of necessary information is whether the presence of *i*NKT cells *per se* is sufficient to exert a protective effect in cancer, to alleviate autoimmune diseases, and to induce transplantation tolerance or whether activation of these cells is required. The stimulation of *i*NKT cells is required to inhibit cancer cell growth and to mitigate autoimmune diseases, but tolerance induction in allogeneic cardiac transplantation does not require activation. Furthermore, there are conflicting results regarding the efficacy of *i*NKT cell stimulation in alleviating disease (Godfrey and Kronenberg 2004; Kronenberg and Rudensky 2005). The third barrier to the clinical application of *i*NKT cells is functional differences between mouse and human *i*NKT cells. In humans, the $CD4^-$ *i*NKT cell subset produces IFN-γ, whereas the $CD4^+$ *i*NKT cell subset produces both Th1 and Th2 cytokines (Gumperz et al. 2002; Lee et al. 2002). Such differential expression of cytokines is not evident in mice.

Until now, our knowledge regarding the function of *i*NKT cells was limited in that neither biochemical nor molecular biological information was available, primarily due to the limited number of *i*NKT cells. Thus the development of novel techniques to study *i*NKT function is required to explore the potential clinical applications of this cell type.

*ESCs prepared via SCNT using NKT cell nuclei*

Derived from the inner cell mass of the blastocyst, ESCs possess the ability to differentiate into all cell types of the body, with the exception of placenta. Unlike somatic stem cells, ESCs expand indefinitely while remaining multipotent. The derivation of *i*NKT cells from ESCs would provide an indefinite number of cells, which would, in turn, greatly facilitate biochemical and molecular analyses of *i*NKT cells. It would also make it possible to prepare naïve *i*NKT cells without being affected by housing conditions that often cause a Th1 or Th2 bias in mice.

Since *i*NKT cells are a subset of T cells, previous studies have attempted to induce these cells by incubating

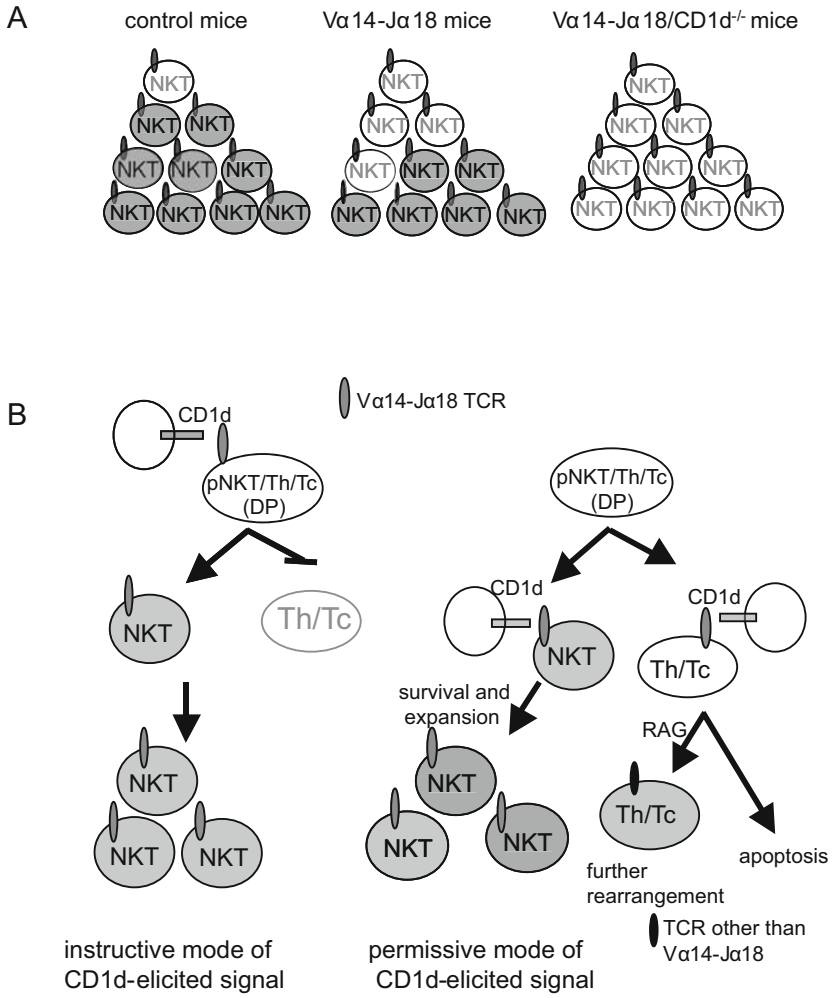


**Fig. 4. A:** Presence of CD1d-reacted and CD1d-nonreacted *i*NKT cells in thymocytes. In control mouse thymocytes (e.g. C57BL/6), most *i*NKT cells are CD1d-reacted (plain text and shaded ovals), whereas few CD1d-nonreacted *i*NKT cells are present (half-tone text and open ovals). In Vα14-Jα18 mice, CD1d-nonreacted *i*NKT cells are present among Vα14-Jα18 thymocytes, as evidenced by the expression profile of the cell surface molecules. In Vα14-Jα18/CD1d$^{-/-}$ mice, no CD1d-reacted *i*NKT cells were present in thymocytes from mice harboring in-frame Vα14-Jα18 in the absence of *CD1d*. **B:** Instructive versus permissive signaling in determining *i*NKT cell fate from multipotent progenitors. In the instructive mode of CD1d-mediated signaling (left panel) in the DP stage, the (Vα14-Jα18TCR)-CD1d signal instructs multipotent progenitor cells (pNKT/Th/Tc) to become *i*NKT cells at the expense of helper T (Th) and cytotoxic T (Tc) cells (half-tone text). Note that neither Th nor Tc cells are generated via (Vα14-Jα18TCR)-CD1d signaling. In the permissive mode of CD1d-mediated signaling (right panel), the (Vα14-Jα18TCR)-CD1d signal supports the survival and proliferation of the committed *i*NKT cells alone. The commitment of these *i*NKT cells is determined independently of the (Vα14-Jα18TCR)-CD1d signal. In this case, simultaneously generated Th and Tc cells that happen to express the Vα14-Jα18 TCR are eliminated via apoptosis. Alternatively, Th and Tc undergo further rearrangement (i.e. become Vα14-Jα18) and develop into *i*NKT cells. Such a mechanism may explain the predominance of *i*NKT cells in Vα14-Jα18 mouse thymocytes.

ESCs prepared from fertilized eggs under the same conditions used to induce T lymphocytes (Schmitt et al. 2004). However, few *i*NKT cells were present among the DP T cells that differentiated from ESCs as judged by staining with α-galactosylceramide (GalCer)-loaded CD1d dimer (Wakao et al., unpublished observation). These results indicate that the generation of *i*NKT cells is not a predetermined event before the onset of T cell ontogeny; rather, it is the result of the random recombination of Vα and Jα during thymocyte development. It is thus highly probable that cells that happen to harbor in-frame rearranged Vα14-Jα18 are permitted to survive/proliferate upon receiving the CD1d-mediated signal. Given that the progeny of a cloned mouse from a NKT cell harboring an in-frame rearranged Vα14-Jα18 in one allele (Vα14-Jα18 mouse) possessed an increased number of *i*NKT cells, ESCs were generated via SCNT using nuclei from NKT cells (ntESCs) and subjected to T lymphocyte differentiation (Fig. 1 and 3B) (Inoue et al 2005; Wakao et al. 2007; Wakao et al. 2008). The co-culture of ntESCs with OP9 cells resulted in B cell generation, whereas the use of OP9 cells ectopically expressing delta-like 1 (OP9-dlk1) culminated in T lymphocyte generation. During ntESC differentiation, T lymphopoiesis charac-

terized by the appearance of the *Rag2*, *Cd3*ε, *Gata3*, and *Vα14-Jα18* transcripts in OP9-dlk1, but not in OP9 cells. Analyses using T lymphocytes with αGalCer-CD1d dimer revealed that more than 90% of the T cells were *i*NKT cells whose TCRβ repertoires were quasi-limited to Vβ8. Subsequent analyses of cell surface markers revealed that these cells were NK1.1$^-$CD69$^-$CD44$^{low}$CD24$^+$, resembling the most immature stage of *i*NKT cells in the thymus (Benlagha et al. 2002; Benlagha et al. 2005). Intriguingly, *i*NKT cells generated from ntESCs were composed of CD8$^+$ and CD4$^+$CD8$^+$ cells. However, few CD4$^+$ *i*NKT cells were present, which is similar to T lymphocytes derived from ESCs (Schmitt et al. 2004). The cytokine profile of these *i*NKT cells was Th2 predominant, although co-culture with CD4$^+$CD8$^+$ cells generated from ESCs drove the production of IFN-γ, suggesting that *i*NKT cells mature via interaction with CD4$^+$CD8$^+$ DP cells. The former observation is consistent with a previous report that immature thymic *i*NKT cells showed a Th2--biased cytokine production profile (Benlagha et al. 2002). T lymphocytes generated from ESCs did not mature on their own after adoptive transfer; rather, maturation *ex vivo* via fetal thymic organ culture (FTOC) was required to reconstitute immune response in immunocompromised mice (Schmitt et al. 2004). Human FTOC, however, is not feasible, making it difficult to apply T lymphocytes in clinical use. In marked contrast, *i*NKT cells generated from ntESCs matured autonomously upon intravenous transfer and exerted an antigen-specific adjuvant effect (Wakao et al. 2008). Invariant NKT cells converted to a mature phenotype, as evidenced by the expression profile of cell surface molecules. Indeed, CD44, CD69, and NK1.1 were concomitantly up-regulated with a decrease in CD24 in both the spleen and liver.

It is noteworthy that *i*NKT cells are also detectable in bone marrow, but not in the thymus upon adoptive transfer, indicating that the homing activity of *i*NKT cells may not be pre-determined in the thymus. Further detailed study is required to elucidate the mechanism underlying homing activity and to identify the molecules involved.

After adoptive transfer, *i*NKT cells become CD4$^-$ CD8$^-$ or CD8$^+$, whereas CD4$^+$CD8$^+$ *i*NKT cells disappear. Given that CD4$^-$CD8$^-$ *i*NKT cells stem from CD4$^+$ *i*NKT cells, the absence of CD4$^+$ *i*NKT cells is somewhat mysterious (Benlagha et al. 2005). It is possible that the differentiation of *i*NKT cells from ntESCs does not mirror what happens in the thymus; alternatively, CD4$^+$ *i*NKT cells may stem from CD4$^-$CD8$^-$ *i*NKT cells. In either case, it is important to note that *in vitro* differentiation of *i*NKT cells directly demonstrates the presence of CD4$^+$CD8$^+$ *i*NKT cells, which are considered to be the most immature cell type in *i*NKT ontogeny. The presence of CD8$^+$ *i*NKT cells has been reported in Vα14-Jα18 mice (Wakao et al. 2007). Since little information is currently available regarding this cell type, further analyses are required to better understand the nature of this novel *i*NKT cell subset.

Maturation in *i*NKT cells upon adoptive transfer is signaled by phenotypic change and the cessation of *Rag* transcription. Invariant NKT cells obtained from ntESCs expressed *Rag,* even though they were capable of producing cytokines, suggesting that these cells are at a stage equivalent to positive selection; further maturation could therefore occur in the periphery or the thymus, depending on the presence of CD1d. Although the endogenous ligand(s) responsible for autonomous maturation are unknown, isoglobotrihexosylceramide (iGB3), a recently reported endogenous ligand, is a likely candidate (Zhou et al. 2004). However, the presence of *i*NKT cells in iGB3 synthetase knockout mice indicates the existence of other endogenous ligands (Porubsky et al. 2007).

*ntESC-derived iNKT cells against cancer*

A subset of IFN-γ-producing CD8$^+$ T cells was shown to be responsible for protective immunity against the liver stage of mouse malaria (Nussenzweig et al. 1967). Subsequent studies showed that the co-administration of αGalCer with irradiated mouse malarial sporozoites or recombinant adenoviruses expressing a malarial antigen, the *Plasmodium yoelii* CS protein, increased the level of protective anti-malarial immunity (Gonzalez-Aseguinolaza et al. 2002). This adjuvant effect of αGalCer is now known to be contingent upon *i*NKT cells, CD1d, and IFN-γ. In terms of innate immunity, the administration of αGalCer induces the maturation of dendritic cells (DCs), which in turn results in IL-12 production. IL-12 then activates NK cells, culminating in the production of IFN-γ and the destruction of MHC I$^{low}$ cells. Activated *i*NKT cells can also produce IFN-γ and kill tumor cells. In contrast, in mice treated with antigen and αGalCer, CD40L in *i*NKT cells triggered DC maturation and the mounting of strong antigen-specific CD4$^+$ and CD8$^+$ T cell responses (Fujii et al. 2007).

Therefore we explored the ability of ntESC-derived *i*NKT cells to elicit this antigen-specific adjuvant effect and found that *i*NKT activation with αGalCer resulted in the enhanced production of IFN-γ from CD8$^+$ T cells, a prerequisite for anti-tumor activity. Consequently, the injection of ntESC-derived *i*NKT cells concomitant with αGalCer resulted in the antigen-specific repression of cancer cell growth (Wakao et al. 2008). These data demonstrate that ntESC-derived *i*NKT cells exert an antigen-specific adjuvant effect, reinforcing the immune reaction. Whether these *i*NKT cells are beneficial for the induction of organ transplantation tolerance or could be used to alleviate autoimmune diseases, such as experimental autoimmune encephalomyelitis and type I diabetes mellitus, requires future study.

## FUTURE DIRECTIONS

The advent of ntESC-derived *i*NKT cells opens new avenues into the development of human *i*NKT cells from ESCs. However, the preparation of ntESCs via SCNT using the nuclei from human *i*NKT cells is a technical hurdle. Moreover, ethical concerns regarding the

use of human eggs remain. Alternatively, it may be possible to produce cells capable of differentiating into *i*NKT cells via the transduction of human ESCs with retroviral vectors expressing Vα24-Jα18 TCR. From the perspective of genetic reprogramming, it may also be feasible to prepare induced pluripotent cells by incubating *i*NKT cells with specific factors (Jaenisch and Young 2008). This will ensure the appropriate genomic configuration (i.e. the presence of in-frame rearranged *Vα24-Jα18* in the TCRα locus), which is a prerequisite for the induction of *i*NKT cells. However, the probability of developing cancer from these cells would not be negligible.

It is also important to consider the use of glycolipids other than αGalCer to modify the cytokine production profile of *i*NKT cells for therapeutic use. For example, OCH, an analog of αGalCer with a shorter aliphatic chain, drives *i*NKT cells toward Th2 cytokine production and alleviates autoimmune disease (Miyamoto et al. 2001; Stanic et al. 2003). By contrast, the use of C-glycoside, another analog of αGalCer, preferentially produces IFN-γ and culminates in superior protection against malaria and melanoma metastases (Schmieg et al. 2003). However, the efficacy of these glycolipids has only been reported in mice; it remains unclear whether these factors will be equally effective in humans. In mice, glycolipid treatment does not consistently produce beneficial results; in some cases, glycolipid treatment exacerbated the disease state. Therefore, further investigation of glycolipids (or mimetics) that control the *i*NKT activity in a disease-dependent manner is critical for the successful use of *i*NKT cells (Kronenberg and Rudensky 2005). Armed with such compounds, ESCs-derived *i*NKT cells may have significant future potential in regenerative medicine or cell-based immunotherapy.

**Acknowledgment:** We thank Dr. K. Iwabuchi for a critical review of this manuscript.

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