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Title: invariant Natural Killer T cell activation following transplantation

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Abstract

Invariant natural killer T (*i*NKT) cells have been shown to play a key role in the regulation of immunity in health and disease. However, *i*NKT cell responses have also been found to influence both rejection and the induction of tolerance following transplantation of allogeneic cells or organs. Although a number of mechanisms have been identified that lead to *i*NKT cell activation, how *i*NKT cells are activated following transplantation remains unknown. This review will attempt to identify potential mechanisms of *i*NKT cell activation in the context of transplantation by applying knowledge garnered from other disease situations. Furthermore, we put forward a novel mechanism of *i*NKT cell activation which we believe may be the dominant mechanism responsible for *i*NKT activation in this setting i.e. bystander activation by IL-2 secreted by recently activated conventional T cells.

Main Text

NKT cells represent a small population of T lymphocytes that are reactive to lipid antigens, presented in the context of the non-polymorphic MHC-class I like molecule, CD1d [1]. Although there appears to be a number of subtypes of NKT cells, which can be determined through their T cell receptor (TCR) usage, cytokine production, expression of specific surface molecules and reactivity, the most extensively characterized are the invariant NKT (*i*NKT) cells [2]. *i*NKT cells are defined by the expression of a semi-invariant TCR composed of a Vα14-Jα18 chain associated with Vβ8.2, Vβ7, Vβ2 chains in mice and Vα24-Jα18 associated with Vβ11 in humans [3-5]. The distribution of *i*NKT cells is varied, accounting for 30% of the total T cell population in the liver and <1% in the spleen, lymph nodes and blood in mice and at slightly lower levels in humans [6, 7]. Although *i*NKT cells represent a relatively low frequency of T cells, their limited TCR diversity means that they respond at high frequency following activation.

*i*NKT cells have been shown to play an important role in health and disease being involved in both the induction of immunity to pathogens and malignancies in addition to the maintenance of tolerance to self-antigens. For example, mice deficient in NKT cells are susceptible to the development of

chemically induced tumours, whereas wild-type mice are protected [8]. Indeed, these experimental findings correlate with clinical data showing that patients with advanced cancer have decreased iNKT cell numbers in peripheral blood [9]. In addition, in studies using Non-Obese Diabetic (NOD) mice that develop a spontaneous form of type 1 diabetes (T1D) mediated by auto-reactive T cells iNKT cells can alter the kinetic of disease onset and severity of disease. In this model, such mice have been found to contain reduced numbers of NKT cells and either activation or increasing the number of iNKT cells in NOD mice affords a degree of protection from T1D [10-12].

The prototypical *i*NKT cell agonist is the marine-sponge derived α-galactosylceramide (α-GalCer), although a broad spectrum of endogenous and exogenous ligands have now been characterised [13-19]. The use of α-GalCer has provided important insight into how *i*NKT cell activation can regulate immunity and collectively these studies have demonstrated that *i*NKT cells occupy an important immunological niche providing a bridge between the innate and adaptive arms of the immune system [2]. For example, *i*NKT cell activation is associated with key steps in ensuring effective immune responses through the maturation of dendritic cells, activation of NK cells and B cells, enhancing T cell responses and suppression of myeloid-derived suppressor cells (MDSC) and IL-10-producing neutrophils [20-29]. Although, many of

these mechanisms have been defined using potent *i*NKT cell agonists, such as α-GalCer, the intricacies of *i*NKT cell activation, in the absence of such super-agonists, have largely been restricted to *i*NKT cell responses in the context of cancer, autoimmunity and pathogenic infections [30-32]. In this review we will discuss the mechanisms of *i*NKT cell activation in these disease situations and then relate these potential modes of activation to *i*NKT cell responses following transplantation.

iNKT cells are activated following transplantation

In mice, the absence of NKT cells does not alter the kinetics of rejection of fully MHC mismatched cardiac, skin or islet (placed under the kidney capsule) transplants [33-35] which may be due to the potent conventional T cell alloimmune response overshadowing any contribution to rejection made by *i*NKT cells. However, there is ample evidence to suggest that *i*NKT cells are activated and participate in responses to transplanted tissue. For example, *i*NKT cells have been shown to infiltrate both cardiac and skin allografts prior to rejection and have been found in expanded numbers in peripheral lymphoid tissue following transplantation [36-38]. Furthermore, an increase in the number of $CD4^+$ *i*NKT cells has been reported in patients undergoing an acute cardiac rejection episode compared to those patients with stable transplants [39].

The impact of iNKT cell activation following transplantation

*i*NKT cells are not only activated but also influence the ensuing immune response to allografts being either pro-rejection or facilitating the induction of tolerance depending on the model studied (Table 1; reviewed in [40]). For example, it has been consistently found that animals deficient in either NKT cells or *i*NKT cells are resistant to the induction of tolerance by costimulatory/coreceptor molecule blockade [33, 38, 41]. Importantly the adoptive transfer of NKT cells into such mice restores tolerance which is dependent on IFNγ, IL-10 and/or CXCL16 [33, 37, 38, 41, 42]. In addition, *i*NKT cells have proved to be essential for the induction of tolerance to corneal allografts and have been demonstrated to prevent graft versus host disease (GVHD) in an IL-4 dependent manner [43-45].

However, following transplantation *i*NKT cell responses do not always promote allograft acceptance as we have shown that the activation of *i*NKT cells can promote the rejection rather than acceptance of skin allografts (Janes SE, unpublished observation). Furthermore, transplantation of islets into the liver via the portal vein resulted in the activation of *i*NKT cells, IFNγ secretion and islet destruction mediated by neutrophils [34, 46]. However, this *i*NKT cell response appears to be unique to intra-liver delivery of islets as the same

response was not seen upon islet transplantation under the kidney capsule [34]. These data may suggest that *i*NKT cells require a degree of inflammation or ischemia to become activated, since Li *et al* has shown that following ischemia reperfusion injury of the kidney *i*NKT cells are rapidly recruited, produce IFNγ and promote neutrophil recruitment [47]. Furthermore, *i*NKT cell responses may alter depending on the type of transplant carried out, for example, following either vascularised (heart) or non-vascularised (skin) grafts, since the alloantigen drains to iNKT cells residing in the spleen or axillary lymph nodes, respectively. Since, Doisne *et al* have shown that NK1.1^{-}*i*NKT cells resident in peripheral lymph nodes preferentially producing IL-17 under inflammatory conditions [48]. Moreover, we have shown that *i*NKT cells produce IL-17 following skin transplantation and promote graft rejection (Janes SE & Jones ND, manuscript in preparation). However, such *i*NKT cell responses can be manipulated as Oh *et al* have shown that manipulating *i*NKT cells to release IL-10, through multiple injection of α-GalCer, can also prolong skin graft survival [37].

In summary, the activation of *i*NKT cells following transplantation of allogeneic cell or tissue transplants can alter the ensuing alloimmune response either resulting in enhanced rejection or, more often than not, the facilitation of the

induction of tolerance. However, how *i*NKT cells are activated in this context remains incompletely understood.

TCR-mediated activation of iNKT cells

*i*NKT cells express a semi-invariant TCR that confers reactivity to glycolipids presented in the context of CD1d, which is the most extensively characterised mechanism of *i*NKT cell activation. However, in response to infection, the immune system relies upon a complex network of signals through the activation of receptors for pathogen-associated molecular patterns, such as the Toll-like receptors (TLRs), expressed on antigen-presentation cells (APC), consequently promoting antigen-specific T cell responses [49]. During such responses *i*NKT cells have been shown to respond through the recognition of microbial–derived lipid antigens, or through APC-derived cytokines following TLR ligation, in combination with and without the presentation of self or microbial–derived lipids (Figure 1).

There are several types of bacterial antigens that can directly stimulate *i*NKT cells when bound to CD1d, for example, *Sphingomonas* spp, *Borrelia burgdorferi and Leishmania donovani*, acting independently of TLR-mediated activation of APC [14-17, 50, 51]. Interestingly, the composition of the cell wall of Gram-

negative *S. capsulata* which lacks the TLR ligand, lipopolysaccharide (LPS) has a combination of immunogenic (e.g. α-glucuronosylceramide) and non-immunogenic glycosphingolipids (e.g. tetrasaccharide-containing glycosphingolipids), the balance of which is proposed to determine the immune response to infection [15, 16, 52].

*i*NKT cell have also been shown to be activated during viral infection as De Santo *et al* demonstrated that both NKT ($CD1d^{-/-}$) and *i*NKT ($J\alpha18^{-/-}$) cell deficient mice were highly susceptible to influenza compared with wild-type mice [26]. In this model *i*NKT cells were found to suppress the expansion of MDSC which were expanded in CD1d and $J\alpha18^{-/-}$ mice [26]. Importantly, although the exactly mechanism of *i*NKT cell activation was not determined, the authors suggest that *i*NKT cells required TCR-CD1d interactions as the adoptive transfer of *i*NKT cells to $J\alpha18^{-/-}$ but not $CD1d^{-/-}$ mice suppressed MDSC expansion following infection with PR8 [26].

In addition to responses to pathogen-derived glycolipids, *i*NKT cells may respond to endogenously produced glycosphingolipids such as isoglobotrihexosylceramide (IGb3) and lysophosphatidylcholine (LPC) as well as further unidentified self non-glycosphingolipids ligands [18, 19, 53-55].

Whilst it appears that in the steady state peripheral *i*NKT cells are not activated by self-glycolipids [56] it has been suggested that TLR signaling attenuates alpha-galactosidase A expression (that degrades potential glycolipid antigens) resulting in the accumulation and enhanced presentation of self-glycolipids and subsequent activation of *i*NKT cells [57]. This pathway for self-glycolipid recognition appears important for NKT activation by pathogens such as *Salmonella enteric* which does not contain agonist glycolipids [58-60]. In such instances endogenous glycolipid ligands may be increased which in addition to the release of APC-derived cytokines, such as IL-12, results in *i*NKT cell activation [58-61].

Although *i*NKT cells have been shown to be involved in both tumour immunosurveillance [62, 63] and the control of autoimmunity [12, 64, 65] few studies have sought to determine how *i*NKT cells may be activated in these diseases. However, Falcone and colleagues showed that by increasing the expression of CD1d on islets (using a β-cell promoter) that type 1 diabetes failed to develop in NOD mice which was attributed to a recruitment of *i*NKT cells to the islets and biased IL-4 cytokine production [66]. Interestingly, in a model of collagen induced arthritis one further pathway of TCR-mediated *i*NKT cell activation has been described in which *i*NKT cells were found to respond to an

endogenous collagen peptide (PPGANGNPGPAGPPG; $mCII_{707-721}$) rather than to a glycolipid [67]. This peptide was shown to be presented by CD1d and resulted in the activation of *i*NKT cells [67]. Since Lui and colleagues were able to successfully demonstrate that $mCII_{707-721}$ vaccination was able to provide benefit in a number of autoimmune diseases (i.e. antigen-induce airway inflammation, collagen-induce arthritis and experimental autoimmune encephalitis), it is possible that TCR-mediated *i*NKT cell activation may be required in the context of autoimmune responses [67].

Taken together these data suggest that following transplantation *i*NKT cells may be activated by glycolipids presented by donor APC or recipient APC that have received inflammatory signals resulting in the presentation of self-glycolipids. However, there is little evidence to support a role for TCR-mediated activation of *i*NKT cells following transplantation. In fact, Oh *et al* have shown that where *i*NKT cells were required for prolonged allograft survival that this process was not dependent on CD1d expression by the donor [37]. We have similarly found that *i*NKT cells expand in lymph nodes following fully allogeneic skin transplantation regardless of whether the donor expresses CD1d (Jukes *et al*, *in press*). Furthermore, the transfer of *i*NKT cells into CD1d knockout mice was found to restore *i*NKT cell mediated graft

prolongation suggesting that *i*NKT cell function in this model was not dependent on recognition of glycolipids presented by recipient APC [37].

iNKT cell activation by cytokines

In contrast to *i*NKT cell activation through direct TCR-mediated signals, following the recognition of glycolipid, *i*NKT cells can also be directly activated by pro-inflammatory cytokines. *i*NKT cells constitutively express the IL-12 receptor and have been shown to respond directly to IL-12 leading to tumour clearance [68]. Furthermore, Nagarajan and Kronenberg found that APC exposed to *Escherichia coli* lipopolysaccharides (LPS) resulted in the release of IL-12 and IL-18 [69]. NKT cells exposed to both IL-12 and IL-18 produced IFNγ thus amplifying innate-derived signals via TLR to promote immunity [69]. Furthermore, NKT responses in the context of Mouse Cytomegalovirus (MCMV) were shown to be dependent on TLR9 dependent activation of DC resulting in the subsequent activation of *i*NKT cells by IL-12 and IFNα/β [70, 71]. Importantly, *i*NKT cell activation and IFNγ secretion were found to be independent of CD1d-glycolipid recognition as *i*NKT cells were similarly activated in the presence of a blocking CD1d mAb or following transfer to $CD1d^{-/-}$ hosts.

Brigl and colleagues have recently reaffirmed this pathway of activation by investigating the dominant signals received by *i*NKT cells following exposure to microbes that contains previously defined *i*NKT cell ligands (*Sphingomonas yanoikuyae, Streptococcus pneumoniae*) [72]. Interestingly, these studies found that following infection *i*NKT cell activation was dominated by activation through the IL-12/STAT-4 pathway rather than through TCR mediated recognition [72].

The process of transplantation results in surgical trauma and ischemia reperfusion injury that results in the activation of DC and the expression of proinflammatory cytokines [73]. Indeed transplantation has been shown to be accompanied by the release of a number of endogenous TLR ligands such as heat shock proteins and oligosaccharides of hyaluronan that can result in TLR4 dependent DC maturation [74, 75]. Therefore, the transplantation process itself may result in the activation of *i*NKT cells by IL-12/IL-18/IFNα/β following TLR ligation of APC. However, although no study to date has addressed this possibility directly, we and others have shown that *i*NKT cells expand in the draining lymph node following allogeneic but not syngeneic transplantation (Jukes *et al, in press; [37]*). Furthermore, *i*NKT cells were found to only have infiltrated allogeneic and not syngeneic skin and heart transplants (Figure 2;

data not shown). We would argue that if *i*NKT cells were activated after transplantation by pro-inflammatory cytokines either directly or in combination with self-glycolipid recognition then activation should have been evident after syngeneic transplantation. This does not rule out the impact of innate derived signals in shaping the ensuing *i*NKT cell response but only that it is unlikely to be the primary driving force behind *i*NKT cell activation following transplantation.

Bystander iNKT cell activation during adaptive immune responses

We and others have been able to reveal that *i*NKT cells are activated following transplantation of allogeneic tissue. This led us to question whether *i*NKT cells may receive activating signals from the adaptive as well as the innate immune system. Although, *i*NKT cells died when cultured with allogeneic stimulator cells we found that the addition of conventional alloreactive T cells to such cultures resulted in *i*NKT cell activation, proliferation and cytokine production both *in vitro* and *in vivo* (Jukes *et al, in press*). The mechanism of *i*NKT cell activation was independent of TCR recognition but rather operated through the sequestration of IL-2 derived from activated T cells (Jukes *et al, in press*).

This study now provides a further pathway by which *i*NKT cells may be activated following transplantation (as well as in the context of cancer immunosurveillance and autoimmunity). This highlights the potential of *i*NKT cells to aid in the process of tolerance or rejection and taken together with our recent data may suggest that the adaptive immune response could simply 'tap' into the reservoir of *i*NKT cell-derived cytokines, to aid in the successful generation of either anti- or pro-inflammatory immune responses.

Although IL-2 may activate *i*NKT cells, clearly the already described innate environmental cues may influence the subsequent behaviour of *i*NKT cells in terms of expansion and cytokine production. Therefore conditions that lead to allograft tolerance may promote the expansion of *i*NKT cells with suppressive potential such as the recently described Foxp3$^+$ [76] or IL-10 producing [41] *i*NKT cells. In particular *i*NKT cells and Treg may unite in depleting the microenvironment of IL-2, encouraging the production of anti-inflammatory cytokines (such as IL-10, TGFβ) thereby promoting allograft survival (Figure 3). Alternatively, if *i*NKT cells respond to IL-2 under pro-inflammatory conditions then immunity may be amplified by the secretion of pro-inflammatory cytokines by *i*NKT cells (Figure 3).

Can iNKT cells aid transplant tolerance?

Since transplant patients remain on life-long immunosuppression to prevent immune mediated rejection it is important to explore multiple avenues of research that may release such a burden of global immunosuppression, whilst maintaining graft function. Our interest in *i*NKT cells is to understand not only how they are involved in the immune response to allografts but also how they may be used as a therapeutic 'stepping-stone' to tolerance. Although, many new synthetic *i*NKT cell agonists have been described which could be used to amplify *i*NKT cell responses following transplantation the factors that govern whether *i*NKT cell activation may promote or attenuate the induction of tolerance are incompletely defined. Understanding not only how *i*NKT cells are activated but also how accessory signals integrate to dictate the class and strength of the *i*NKT cell response will be critical in aiding the development of potential therapeutic strategies involving the manipulation of these cells. Although, there are many questions remaining regarding *i*NKT cell responses following transplantation, dissecting how *i*NKT cells respond in different microenvironments may allow us to 'tap' into the potential ability of *i*NKT cells to amplify pathways that promote tolerance.

A

Species	Transplant	Tolerance Regimen	Mechanism of action	Ref.
Mice	Heart	Anti-LFA-1/ICAM-1 or Anti-B7-1/B7-2	IFNγ production?	[33]
		Anti-CD154 mAb	*i*NKT cells require CXCL16/CXCR6 interactions to traffic to allograft	[38]
		Anti-CD154 mAb	IL-10 production	[41]
Mice	Skin	None	IL-10 production	[37]
Mice	Heart	Anti-CD4, CD8, CD154 mAb	Not determined	*
Mice	Islet (rat xenograft)	Anti-CD4 mAb	IFNγ production but the dose of CD4 is critical for tolerance induction.	[42]
Mice	Cornea	None	Enhanced regulatory T cell function	[43]
Mice	Bone Marrow	None	Prevent GvHD. IL-4 dependent.	[44, 45]

B

Species	Transplant	Tolerance Regimen	Mechanism of action	Ref.
Mice	Islet	None	IFNγ production promotes PMN recruitment to graft	[46]
		None	Islet destruction only found when Islets injected into portal vein	[34]
		Rapamycin	Not determined	[34]
Mice	Skin	Anti-CD4, CD8, CD154 mAb	Not determined	*
Mice	Skin	None	Induction of a Th17 response	**

Table. 1 Summary of the involvement of *i*NKT cells in transplant tolerance (A) and rejection (B). It should be noted that in one study in Islet transplantation *i*NKT cells were not shown to contribute to either transplant tolerance or rejection [35]. GvHD = Graft versus host disease. **Jukes J-P, Zhao Z and Jones ND unpublished observations. ** Janes SE and Jones ND, manuscript in preparation.*

Figure 1. *i*NKT cells can become activated through a number of directly or indirect pathways. The 'direct' pathway acts through the recognition of synthetic or microbial ligands presented in the context of CD1d. In contrast the 'indirect' pathways relies upon the release of cytokines from antigen presenting cells (APC) that have been activated through TLR ligation in the presence or absence of recognition of CD1d-self-glycolipid complexes. In addition, *i*NKT cell-APC interactions can be influenced by ligation of a number of co-stimulatory molecules, for example, CD28, CD40L, OX40, GITR, ICOS, 41BB (CD137).

Figure 2. iNKT cells are recruited to allogeneic but not syngeneic skin and heart grafts in multiple mouse strain combinations. CBA (syngeneic) or C57BL/10 (allogeneic) skin grafts were analysed by quantitative real-time PCR (qRT-PCR) for Vα14Jα18 mRNA expression (to quantify the infiltration of iNKT cells) on day 3, 5 and 10 post transplant (A). In addition, heart grafts taken from B6 ($H2^b$) recipients of either syngeneic (C57BL/6) or allogeneic (BALB/c) grafts were analysed for the expression of Vα14Jα18 mRNA on day 1, 3 and 5 post transplant (B). Results are expressed as mRNA (Units/HPRT) with naïve skin or

heart used to give baseline expression. n=3 per time point. *All error bars indicate mean ± standard deviation.*p=<0.05.*

Figure 3. Mechanisms by which *i*NKT cells are activated following transplantation. *i*NKT cells have been implicated in both the induction of transplantation tolerance and rejection although the mode of activation remains largely unknown. We have recently described a novel mechanism of bystander *i*NKT cell activation following sequestration of IL-2 produced by activated conventional T cells (dotted black arrow). This pathway of activation is independent of CD1d-glycolipid/TCR interactions and is associated with the production of effector cytokine release (i.e IFNγ). We hypothesis that IL-2 mediated *i*NKT cell activation may be important in creating a microenvironment that promotes transplant tolerance, as Treg and *i*NKT cells may synergistically act to deplete IL-2 from the local microenvironment in addition to suppressing alloreactive T cell responses via the release of anti-inflammatory cytokines and/or interaction with APC bearing alloantigen. It is conceivable that such responses may be further enhanced through *i*NKT cell-APC interactions via cytokine production (i.e. IL-12) and co-stimulatory molecules (CD28, CD40L, OX40, GITR, ICOS, 41BB).

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