Adaptability of the semi-invariant natural killer T-cell receptor towards structurally diverse CD1d-restricted ligands

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The semi-invariant natural killer (NK) T-cell receptor (NKTcr) recognises structurally diverse glycolipid antigens presented by the monomorphic CD1d molecule. While the α -chain of the NKTcr is invariant, the β -chain is more diverse, but how this diversity enables the NKTcr to recognise diverse antigens, such as an α -linked monosaccharide (α -galactosylceramide and α -galactosyldiacylglycerol) and the β -linked trisaccharide (isoglobotriaosylceramide), is unclear. We demonstrate here that NKTcrs, which varied in their β -chain usage, recognised diverse glycolipid antigens with a similar binding mode on CD1d. Nevertheless, the NKTcrs recognised distinct epitopic sites within these antigens, including α -galactosylceramide, the structurally similar α -galactosyldiacylglycerol and the very distinct isoglobotriaosylceramide. We also

show that the relative roles of the CDR loops within the NKTcr β -chain varied as a function of the antigen. Thus, while NKTcrs characteristically use a conserved docking mode, the NKTcr β -chain allows these cells to recognise unique aspects of structurally diverse CD1d-restricted ligands.

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Introduction

The antigen receptor of $\alpha\beta$ T cells faces a daunting recognition problem because each T-cell receptor (TCR) interfaces multiple ligands during its lifetime and yet maintains specificity. They recognise self-ligand (peptide or lipid) in the context of a self-antigen-presenting molecule (major histocompatibility complex (MHC) class I, class II or CD1) in the thymus; foreign ligand in the context of a self-MHC/CD1 molecule in the periphery; and in some cases, non-self-ligands in the context of non-self-MHC molecules. This recognition conundrum is much more daunting for the semi-invariant natural killer (NK) T-cell receptor (NKTcr) because its recognition landscape is predominantly germline-encoded and, hence, highly conserved (Borg *et al*, 2007; Scott-Browne *et al*, 2007; Wun *et al*, 2008), while the antigenic landscapes of CD1d-lipid complexes are highly diverse (Koch *et al*, 2005; Zajonc *et al*, 2005, 2008; Kinjo *et al*, 2006). Understanding how NKT cells see extremely diverse lipid antigens is important because this innate T lymphocyte regulates early immune responses to pathogens and tumours, and is a target for immunotherapy against some autoimmune diseases (Van Kaer, 2005).

The TCR α and β -chains each comprise variable (V) and constant (C) domains, in which the V domains contain the complementarity determining region-1 (CDR1), CDR2 and CDR3, that interact with the peptide-laden MHC (pMHC) molecules. While variations within V-gene segments confer diversity to CDR1 and CDR2, imprecise joining and non-templated nucleotide additions during V(D)J recombination generate the much more diverse CDR3 (Rudolph *et al*, 2006). TCR specificity is not limited to peptidic antigens. NKT cells express a semi-invariant TCR that recognises lipid-based antigens presented by the monomorphic CD1d molecule. The NKTcr is made up of an invariant TCR α -chain, which is generated by TRAV11*02 (mouse and rat V α 14) to TRAJ18 (J α 18) or orthologous TRAV10 (human V α 24) to TRAJ18 rearrangement. Despite the stochastic nature of V(D)J recombination, stringent intrathymic selection steps permit only one CDR3 α for NKT cells. Thus, NKT cells express a completely invariant TCR α -chain that is highly conserved across

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species (Koseki *et al*, 1990; Porcelli *et al*, 1993; Dellabona *et al*, 1994; Lantz and Bendelac, 1994). In contrast, CDR3 β of TRBV13-2*01 (mouse V β 8.2) and the orthologous TRBV25-1 (human V β 11), which pair with the invariant V α 14 and V α 24 α -chains, respectively, are highly diverse (Porcelli *et al*, 1993; Lantz and Bendelac, 1994). So too are the CDR3 β of TRBV29*02 (V β 7) and TRBV1 (V β 2) β -chains, which also pair with V α 14 and V α 24 α -chains in mice (Lantz and Bendelac, 1994; Capone *et al*, 2003). Thus, NKT cells have an invariant TCR α -chain, but maintain some diversity in their TCR β -chain, which is why they are referred to as semi-invariant.

The NKTCr binds structurally diverse CD1d-restricted ligands that range from α -linked monohexosylceramides such as the prototypic NKT-cell antigen α -galactosylceramide (α GalCer; Kawano *et al*, 1997; Burdin *et al*, 1998) and the closely related α -galacturonosylceramide (α GalACer; *Sphingomonas* spp.; Kinjo *et al*, 2005; Mattner *et al*, 2005; Sriram *et al*, 2005); α -galactosyldiacylglycerol (α GalDAG; *Borrelia burgdorferi*) (Kinjo *et al*, 2006), to the trihexosylceramide isoglobotriaosylceramide (iGb3) Zhou *et al*, 2004). Despite this apparent diversity, most mammalian glycolipids are not recognised by the NKTCr, including lactosylceramide (LacCer), globotriaosylceramide (Gb3; α 1,4Gal-LacCer), which is closely related to iGb3 (α 1,3Gal-LacCer), monosialylganglioside GM1 (α 2,3sialyl-LacCer) or isoglobotetraosylceramide (iGb4) (Zhou *et al*, 2004). A small subset of NKTCrs also recognise phosphatidylethanolamine (Rauch *et al*, 2003), phosphatidylinositol (Gumperz *et al*, 2000), β -galactosylceramide (Ortaldo *et al*, 2004; Parekh *et al*, 2004), the disialylganglioside GD3 (Wu *et al*, 2003) and microbial phosphatidylinositol-mannoside (PIM₄; *Mycobacterium tuberculosis* (Fischer *et al*, 2004). Crystal structures of several CD1d-antigen complexes showed that these lipids bind to CD1d through their long-chain base (LCB) and/or fatty acyl chain(s) so that the polar head groups protrude out of the CD1d antigen-binding site (ABS). Furthermore, the disposition of the head group depends on the unique pattern of hydrogen-bond network formed by residues of CD1d α 1 and α 2-helices with the polar aspects of the bound ligand (Giabbai *et al*, 2005; Koch *et al*, 2005; Zajonc *et al*, 2005, 2006). Thus, the α -linked galactose of α GalCer lies just above the entrance, flat and parallel to the plain of CD1d's ABS (Koch *et al*, 2005; Zajonc *et al*, 2005). In striking contrast, the β -anomeric linkage of the first sugar orients the glycan α 1,3Gal- β 1, 4Gal- β 1,1'Glc of iGb3 perpendicular to the ABS (Zajonc *et al*, 2008). How the semi-invariant NKTCr recognises multiple diverse ligands in the context of CD1d remains an enigma.

A major advance in understanding how the NKTCr recognises its cognate antigen came from the crystal structure of the human NKTCr/CD1d- α GalCer co-complex (Borg *et al*, 2007). It showed that the V α 24 NKTCr interfaces CD1d- α GalCer in an unusual parallel docking mode by engaging germline-encoded CDR1 α , CDR3 α and CDR2 β residues above the F' pocket (Borg *et al*, 2007; Wun *et al*, 2008). Further, alanine-scanning mutagenesis of mouse and human NKTCrs revealed that they interact with CD1d- α GalCer similarly by using germline-encoded hotspots within CDR1 α , CDR3 α and CDR2 β (Scott-Browne *et al*, 2007; Wun *et al*, 2008). One of these studies also revealed that the V α 14 NKTCr bound to CD1d- α GalCer and the very different CD1d-iGb3 landscapes in a conserved manner, using similar germline-encoded hotspots (Scott-Browne *et al*, 2007). The NKTCr showed

very little conformational change upon ligation (Gadola *et al*, 2006; Kjer-Nielsen *et al*, 2006; Borg *et al*, 2007; Zajonc *et al*, 2008), consistent with a 'lock and key' recognition model where neither temperature nor ionic strength affected the CD1d- α GalCer interaction (Cantu *et al*, 2003). These findings suggest that the NKTCr acts like a pattern-recognition receptor and also explain why the NKTCr ligand recognition is CD1d-restricted. Yet, these studies provide little insight into ligand specificity and the molecular features of the diverse epitopes recognised, and do not explain the basis for distinct TCR β -chain usage for antigen recognition by the NKTCr.

As an approach to understand the role of β -chain in CD1d-restricted antigen recognition, we probed a panel of NKT-cell-derived hybridomas that express different TCR β -chains, as well as a panel of NKTCr point mutants that have altered CDR, with a variety of CD1d-restricted glycolipid ligands and variants with modifications of their sugar moieties. From the recognition patterns that emerged, we concluded that the NKTCr was highly adaptable, depending on the β -chain used, to recognise unique aspects of structurally diverse ligands within a conserved binding footprint on CD1d molecules.

Results

TCR β -chain usage dictates diverse glycolipid antigen recognition by the NKTCr

Unlike the CDR3 α of the NKTCr, the CDR3 β is highly diverse, both in length and primary structure (e.g., Supplementary Table S1). Additionally, CDR1 β and CDR2 β of distinct β -chains differ from each other. Because mouse NKTCr use multiple different β -chains (V β 8.2, V β 7 and V β 2, as well as V β 14 and V β 6; Porcelli *et al*, 1993; Lantz and Bendelac, 1994), we hypothesised that differences within CDR1 β , CDR2 β and CDR3 β account for the ability of NKTCr to recognise diverse lipid antigens. Using a panel of NKT hybridomas expressing identical V α 14 α -chain paired with four commonly used β -chains (V β 8.2, V β 14, V β 7 and V β 6; Lantz and Bendelac, 1994; Gui *et al*, 2001), we determined whether TCR β -chain usage influences recognition of diverse glycolipid ligands (Supplementary Figure S1A). Note that V β 8.2, V β 14, V β 7 and V β 6 have diverse CDR1 β , but partly conserved, CDR2 β sequences (e.g., Y46 and/or Y48 residues that were critical for interaction with the α 1-helix of CD1d; Borg *et al*, 2007; Scott-Browne *et al*, 2007; Wun *et al*, 2008; Mallevaey *et al*, 2009; Pellicci *et al*, 2009). Additionally, the NKT hybridomas expressed diverse CDR3 β regions in association with the same V β -region and, hence, the same CDR1 β and CDR2 β (Supplementary Table S1).

Bone marrow-derived dendritic cells (BMDCs) pulsed with α GalCer, iGb3 or α GalDAG were co-cultivated with the above NKT hybridoma panel and IL-2 secretion was assessed as a measure of activation. The data showed that the V β 8.2+, V β 14+ and V β 7+ hybridomas recognised α GalCer whereas the V β 6+ hybridoma did not (Figure 1A), despite the fact that these hybridomas showed comparable response to antigen-independent stimulation through CD3 ϵ (Figure 1D). These data are consistent with previous reports, which demonstrated that several NKTCr β -chains are permissive to α GalCer recognition (Mallevaey *et al*, 2009). The V β 8.2J β 2.6+ hybridoma was autoreactive (i.e., in the

absence of exogenously added lipid) to BMDCs and was included as a positive control for the assay (Figure 1C).

Compared with the α GalCer recognition pattern, the recognition of iGb3 and α GalDAG by this hybridoma panel was clearly distinct (Figure 1). With the exception of the V β 8.2J β 2.2+ hybridoma, most V β 8.2+ NKT hybridomas recognised iGb3 (Figure 1B). While the V β 7+ hybridoma

also recognised iGb3, the V β 14+ and V β 6+ hybridomas did not (Figure 1B). Note that a titration experiment showed that the hybridoma panel required a minimum of 10 μ g of iGb3 and 20 μ g of α GalDAG for recognition (data not shown). Therefore, because micelle formation and bioavailability influence lipid antigen presentation at higher concentrations, the functional avidity of the different V β -expressing NKTCrs for iGb3 and α GalDAG could not be determined.

As the aforementioned experiment used phytosphingosine-containing iGb3 (Supplementary Figure S1A), we determined whether the chemical make-up of the LCB influenced the recognition pattern. iGb3 made up of sphingosine-, sphinganine (4,5-dihydro)- and 4,5-dibromosphinganine-containing LCB were recognised just as well as phytosphingosine-based iGb3 (data not shown), suggesting that the chemical composition of the sphingoid backbone had very little influence on iGb3 recognition. Finally, all V β 8.2+, the V β 7+ and only the V β 14J β 1.2i+ hybridomas recognised α GalDAG (Figure 1C), whereas the remaining three V β 14+ and the V β 6+ hybridomas were unresponsive to this antigen (Figure 1C).

The varied antigen-recognition pattern could have resulted from variation in TCR expression and differences in the activation threshold of the different NKT hybridomas. Therefore, we determined the IL-2 response of each NKT hybridoma to antigen-independent stimulation using anti-CD3 ϵ monoclonal antibody (mAb). All hybridomas responded similarly to this stimulation (Figure 1D). This result suggests that the recognition pattern of NKT hybridomas was intrinsic to the NKTCr and not due to variation in TCR expression levels or activation threshold. Taken together, the above data suggest that CDR3 β together with CDR1 β and CDR2 β dictate NKTCr's ability to recognise structurally diverse ligands. Alternatively, although less likely, other cell-surface molecules differentially expressed by the hybridomas could result in the observed differences in responses.

V β usage impacts the recognition of 3' and 4' α GalCer analogues

To gain insight into how the NKTCr recognises α GalCer, we used α GalCer analogues, which contain modifications at the 3'- and 4'-hydroxyls of galactose (Supplementary Figure S1B). Note that the α GalCer analogues contained C₈ acyl chain, which does not alter the conformation of CD1d upon binding

Figure 1 TCR β usage impacts V α 14 TCR antigen recognition. (A) BMDCs pulsed with 0.1–100 ng/ml α GalCer overnight were co-cultivated with a panel of NKT hybridomas expressing V α 14 α -chain paired with unique TCR V β -chains (Supplementary Table S1). After 24 h, ELISA measured IL-2 secreted into the culture medium in response to NKT hybridoma activation. The data are represented as the half-maximal response (top panel) or half-maximal stimulatory concentration (EC₅₀; bottom panel) of α GalCer. (B, C) BMDCs pulsed with 10 μ g/ml iGb3 (B) or 20 μ g/ml α GalDAG (C) overnight were co-cultivated with the hybridoma panel and activation was measured by ELISA after 24 h stimulation. (D) NKT hybridomas expressing different β -chains were stimulated with 0.1–27 μ g (in three-fold dilution) plate-bound anti-CD3 ϵ mAb. After ~24 h, IL-2 ELISA was performed on culture supernatants to determine the sensitivity of each hybridoma to direct TCR stimulation. The data are represented as the EC₅₀ of anti-CD3 ϵ mAb. Data are representative of duplicate (B–D) or triplicate (A) experiments, each performed in triplicate.

(Zajonc *et al*, 2005; Pellicci *et al*, 2009). The 3'- and 4'-hydroxyls of α GalCer interact with NKTCr's CDR1 α and/or CDR3 α residues (Supplementary Figure S2) (Pellicci *et al*, 2009) yet their deoxy forms were reported not to affect recognition (Wun *et al*, 2008). Therefore, we determined whether chemical modifications at the 3' (-amino, -azido and -N-acetyl) and 4' (-O-methyl, -O-ethanol and -N-acetyl) of galactose are recognised by NKTCr, and whether V β usage influences this recognition using the approach described above. Replacement of the 3'-hydroxyl with an -amino, -azido or -N-acetyl group prevented recognition by the NKT hybridoma panel regardless of the NKTCr's β -chain composition (Figure 2A). Therefore, although the 3'-hydroxyl itself is dispensable (Wun *et al*, 2008), chemical substitutions at this position are detrimental for NKTCr recognition.

On the other hand, the recognition of the 4' α -GalCer analogues was quite varied. All V β 8.2+ hybridomas recognised these α GalCer variants, but to different degrees, indicating that CDR3 β sequences might influence this recognition (Figure 2B, top row panel 1). The V β 8.2+ hybridomas recognised 4'-O-methyl and 4'-O-ethanol α GalCer analogues to a similar extent (Figure 2B, top row panels 2 and 3), whereas the 4'-N-acetyl variant was less antigenic when compared with the recognition of α GalCer (Figure 2B, top row panel 4). All V β 14+ NKT hybridomas recognised α GalCer but not to the same extent as the V β 8.2+ hybridomas (Figure 2B, middle row panel 1). Recognition of the 4' analogues by V β 14+ NKT hybridomas was J β -dependent, such that only the V β 14J β 2.6+ hybridoma recognised both 4'-O-methyl and 4'-O-ethanol analogues, whereas the V β 14J β 1.2i+ hybridoma recognised only the 4'-O-ethanol variant (Figure 2B, middle row panels 2 and 3). The 4'-N-acetyl variant was not recognised by any of the V β 14+ hybridomas (Figure 2B, middle row panel 4). Furthermore, the V β 7+ NKT hybridoma recognised α GalCer and each of the 4' variants to similar extent, whereas the V β 6+ hybridoma failed to respond to any of these lipid antigens (Figure 2B, bottom row). These data suggest that the 4'-N-acetyl variant is a less potent antigen than the 4'-O-methyl and -O-ethanol analogues, thereby revealing an agonistic hierarchy in NKTCr ligands: α GalCer > 4'-O-methyl $\sim$ 4'-O-ethanol $\gg$ 4'-N-acetyl. As the 4'-N-acetyl analogue is not recognised by V β 14+ hybridomas (Figure 3B), we conclude that the recognition of weak NKTCr ligands is influenced not only by CDR3 β but also by CDR1 β and CDR2 β loops.

V β usage also impacts iGb3 recognition

Low affinity of the CD1d-iGb3/NKTCr interaction has complicated structural, biochemical and functional studies of this ligand-receptor complex (Zhou *et al*, 2004; Zajonc *et al*, 2008). We approached the structural analyses of this complex using a combination of iGb3 variants and the aforementioned hybridoma panel expressing V α 14 α -chain paired with V β 8.2, V β 14, V β 7 or V β 6 β -chain. For this, 2''', 3''', 4''' or 6''' deoxy variants of the terminal galactose were incorporated into the phytosphingosine-based ceramide backbone during syntheses (Supplementary Figure S1C).

As described above, we found that only V β 7+ and V β 8.2+, with the exception of V β 8.2J β 2.2+, NKT hybridomas recognised iGb3 (Figure 1A), a pattern similar to 4'-N-acetyl- α GalCer recognition (Figure 2B). This recognition required the 2'''-hydroxyl group within the terminal galactose

by most (V β 8.2J β 2.5, V β 8.2J β 2.2, V β 8.2J β 2.4 and V β 8.2J β 2.2), and the 3'''-hydroxyl by some (V β 8.2J β 2.5 and V β 8.2J β 2.2 only), V β 8.2 NKT hybridomas (Figure 3). None of the V β 14+ hybridomas recognised iGb3 or its deoxy variants (Figure 3). Therefore, despite their obvious structural differences, the three CDR β loops impact iGb3 recognition in a manner similar to 4'-N-acetyl α GalCer recognition.

CDR1 β and CDR3 β residues, in addition to previously identified 'hotspots' influence the recognition of α GalCer variants

The crystal structure of human CD1d- α GalCer/V α 24-V β 11 NKTCr (Borg *et al*, 2007), as well as mouse CD1d- α GalCer/V α 14-V β 8.2 and CD1d- α GalCer/V α 14-V β 7 NKTCrs (Pellicci *et al*, 2009), combined with mutagenesis studies (Scott-Browne *et al*, 2007; Wun *et al*, 2008; Mallevaey *et al*, 2009), have shown that CDR1 α , CDR3 α and CDR2 β residues are critical for CD1d- α GalCer recognition. As the 4'- α GalCer analogues were differentially recognised by different hybridomas (Figure 2), these variants were used in combination with a panel of V α 14J α 18/V β 8.2J β 1.1-derived mutants with individual amino-acid alteration within their CDRs to determine the CDR β residues that influence recognition of the variant part of the antigen. The rationale behind this approach was that some of the V α 14 NKTCr mutants may gain recognition and reveal the CDR residues that influence recognition either directly or indirectly. From the V α 14-V β 8.2 NKTCr/CD1d- α GalCer crystal structure (Pellicci *et al*, 2009), we were able to precisely gauge whether the effect of the alanine-scan mutants were likely a result of adverse affect on the conformation of the NKTCr (indirect effects, namely CDR1 α : V26A, P28A, H31A, L32A, R33A; CDR1 β : H27A, N29A and CDR2 β : S47A, G49A) or on CD1d-antigen recognition (direct effects). Also note that none of the mutants become autoreactive to BMDCs, the APC used for antigen presentation (data not shown).

As reported previously (Borg *et al*, 2007; Scott-Browne *et al*, 2007; Wun *et al*, 2008), our results revealed that CDR1 α , CDR3 α and CDR2 β residues were critical for α GalCer recognition (Figure 4). For the most part, residues critical for α GalCer recognition were also critical for 4'-O-methyl- and 4'-O-ethanol- α GalCer recognition (Figure 4), suggesting that the footprint of the NKTCr binding to CD1d molecules remains essentially the same for these two variants. Exceptions were CDR1 α mutants R33A, which did not recognise 4'-O-methyl or 4'-O-ethanol- α GalCer, and H31A, which did not recognise 4'-O-methyl- α GalCer, but partially recognised 4'-O-ethanol- α GalCer (Figure 4). The effects of H31A and R33A are perhaps indirect because they are thought to alter the fold of the NKTCr (Borg *et al*, 2007; Scott-Browne *et al*, 2007; Wun *et al*, 2008; Pellicci *et al*, 2009).

The 4'-N-acetyl variant was poorly ($\sim$ 20%) recognised by V α 14J α 18/V β 8.2J β 1.1 TCR compared with recognition of α GalCer (Figures 2B and 4), but alteration of residues within CDR1 α , CDR2 α , CDR1 β and CDR3 β restored recognition of this variant (Figure 4). Specifically, CDR1 α mutants D29A and N30A, as well as CDR2 α mutants V50A, D51A and D54A, overcame poor recognition of the 4'-N-acetyl variant (Figure 4). Similarly, CDR1 β mutants N26A and N29A and CDR3 β mutant G94A also rescued defective recognition of the 4'-N-acetyl analogue (Figure 4). Note that the differential recognition pattern of the mutant NKT hybridomas was not

Figure 2 NKTCrs recognise 4'-hydroxy variants in a distinct manner. **(A)** BMDCs pulsed with 500 ng/ml of 3'-hydroxy variants (Supplementary Figure S1B) overnight were co-cultivated with the same panel of NKT hybridomas described in Figure 1. After 24 h, ELISA measured IL-2 secreted into the culture medium in response to NKT hybridoma activation. **(B)** BMDCs were pulsed with the indicated concentrations of αGalCer or its 4'-hydroxy variants and used to stimulate NKT hybridomas. Top row: Vβ8.2Jβ1.1 (circles); Vβ8.2Jβ2.1 (diamonds); Vβ8.2Jβ2.5 (triangles) and Vβ8.2Jβ2.6 (squares); middle row: Vβ14Jβ1.2i (circles); Vβ14Jβ1.2ii (diamonds); Vβ14Jβ2.5 (triangles) and Vβ14Jβ2.6 (squares); bottom row: Vβ7 (diamonds) and Vβ6 (squares). ELISA measured IL-2 secreted into the culture medium in response to NKT hybridoma activation. **(C)** Schematic rendition of NKTCr recognition pattern. Amino-acid sequence of CDR1β, CDR2β and CDR3β (upper case) of each Vα14 TCR is indicated on the left; lowercase indicates residues flanking each CDR. Vβ sequences were obtained from IMGT (<http://www.imgt.org/textes/IMGTrepertoire/>). Data in panels A and B are representative of two independent experiments performed in triplicate.

due to differences in TCR expression levels or activation thresholds because they were similar for all hybridomas as previously reported (Scott-Browne *et al*, 2007). Together, these data suggest that the family of NKTCrs is afforded plasticity through different TCR β-chains, which, therefore,

permit binding of structurally variant glycolipid ligands. Furthermore, in addition to the CDR1α, CDR3α and CDR2β residues that form a common NKTCr footprint (Scott-Browne *et al*, 2007; Wun *et al*, 2008; Mallevaey *et al*, 2009; Pellicci *et al*, 2009), recognition of weak agonists such as 4'-N-acetyl-

Figure 3 NKTCr interfaces CD1d-iGb3 with a distinct recognition logic. (A) BMDCs pulsed with 10 µg/ml iGb3, 2''-deoxy-iGb3, 3'''-deoxy-iGb3, 4'''-deoxy-iGb3 or 6'''-deoxy-iGb3 were used to stimulate a panel of NKT hybridomas described in Figure 1. After 24 h, ELISA measured IL-2 secreted into the culture medium in response to NKT hybridoma activation. Data are representative of two independent experiments performed in triplicate. (B) Schematic rendition of NKTCr recognition pattern. Amino-acid sequence of CDR1β, CDR2β and CDR3β (upper case) of each Vα14 TCR is indicated on the left; lowercase indicates residues flanking each CDR (for sequences see <http://www.imgt.org/textes/IMGTrepertoire/>).

αGalCer may be influenced by CDR residues outside these 'hotspots'. Consistent with this, the β-chain of the Vβ7 + and Vβ6 + NKTCrs was shown to have a greater role in interacting with CD1d-αGalCer in comparison with the Vβ8.2 + NKTCr (Mallevey *et al*, 2009; Pellicci *et al*, 2009).

CDR2α, CDR1β and CDR3β residues impact on iGb3 recognition

The NKTCr mutant hybridomas were probed with iGb3 and its four deoxy analogues (Supplementary Figure S1C), focusing on the 2''- and 3'''-deoxy variants that were poorly recognised by the Vα14Jα18/Vβ8.2Jβ1.1 NKTCrs (Figure 5). In agreement with a previous study (Scott-Browne *et al*, 2007), we also observed that iGb3 recognition involved the same germline-encoded 'hotspots', which include residues within CDR1α, CDR3α and CDR2β (Figure 5). Furthermore, mutations within all three β-chain CDRs also affected recognition of iGb3, none of which, except CDR3β G94A, were rescued by the 2''- and 3'''-deoxy variants (Figure 5). Most interestingly, alanine mutations either partially (CDR2α V50A) or fully (CDR2α K53A and CDR3β G94A) rescued the recognition of 2''-deoxy-iGb3 and 3'''-deoxy-iGb3, which were poorly recognised by the wt NKTCr (Figure 5). From these data we conclude that iGb3 recognition by the NKTCr is distinct from

αGalCer but shares some characteristics with that of 4'-N-acetyl-αGalCer. Furthermore, the data suggest a potential role of CDR1β and CDR3β, in addition to the CDR2α, loop in the recognition of iGb3.

CDR2α and CDR1β residues impact on αGalDAG recognition

Having determined that the αGalDAG recognition pattern by naturally occurring Vα14 + hybridomas was distinct from that of αGalCer and iGb3 (Figure 1), we tested how the Vα14 NKTCr mutants recognise this antigen. The responses of the mutant hybridomas again revealed that αGalDAG recognition involved the germline-encoded 'hotspots' within CDR1α, CDR3α and CDR2β (Figure 6). While none of the CDR3α mutants recognised αGalCer or iGb3, we found that CDR3α D93A uniquely recognised αGalDAG (Figures 4–6). Further, although Y48A mutant does not recognise the three ligands, the remainder of CDR2β residues were differentially required for recognition: none were required for αGalCer, but S47, G51 and S52 were required for iGb3 while S47 and S52 were required for αGalDAG recognition (Figures 4–6). A similar discordance was also observed with CDR3β mutants: none were critical for αGalCer and αGalDAG recognition (Figures 4 and 6), but many (S93, T95, T96 and T98) were critical for

Figure 4 The 4'-N-acetyl α GalCer variant interacts with V α 14 TCR with a unique register. (A) BMDCs pulsed with 500 ng/ml α GalCer, 4'-O-methyl, 4'-O-ethanol or 4'-N-acetyl variants were used to stimulate a panel of NKTCr mutants consisting of a single point mutation within CDR1, CDR2 and CDR3 of both TCR α - and β -chains. The V α 3.2 + NKT hybridoma was used as the negative control. After 24 h, ELISA measured IL-2 secreted into the culture medium in response to NKT hybridoma activation. As the wt hybridoma and the derived mutants were equally sensitive to antigen-independent stimulation (Scott-Browne *et al*, 2007), the data were normalised to wt IL-2 response to α GalCer-pulsed BMDCs and represented as mean $\pm$ s.e.m. of three independent experiments performed in triplicate. (B) Schematic rendition of NKTCr recognition pattern.

iGb3 recognition (Figure 5). Moreover, CDR2 α residues V50 and K53, which were required for 2'''-deoxy iGb3 recognition, along with D54, were critical for α GalDAG recognition, but were irrelevant for α GalCer recognition (Figures 4–6). These data indicate that the galactose head group of α GalDAG when bound to CD1d is disposed differently from the galactose of α GalCer. Thus, while maintaining a common NKTCr footprint mediated by the 'hotspots', effective recognition of α GalDAG involves different contact residues within these hotspots, and additionally requires CDR2 α and CDR1 β residues.

Discussion

Our current knowledge of how NKTCr recognises its cognate ligands is based on the crystal structure of V α 24–V β 11 NKTCr/CD1d– α GalCer (Borg *et al*, 2007) as well as the recently solved crystal structures of V α 14–V β 8.2 and V α 14–V β 7 NKTCr/CD1d– α GalCer (Pellicci *et al*, 2009) complexes. Moreover these structures have been complemented by mutational studies on the V α 14 and V α 24 NKTCrs (Borg *et al*, 2007; Scott-Browne *et al*, 2007; Wun *et al*, 2008; Mallevaey

Figure 5 NKTCr recognises CD1d-iGb3 with a distinct recognition logic. **(A)** BMDCs were pulsed with iGb3 or its variants (as described in Figure 3A) and used to stimulate a panel of NKTCr mutants, and activation was monitored (as described in Figure 3A). Data were normalised as in Figure 4A and represented as mean $\pm$ s.e.m. of at least three independent experiments performed in triplicate. **(B)** Schematic rendition of NKTCr recognition pattern.

et al, 2009). Collectively, these structures have revealed a similar docking mode between the Vα14-Vβ8.2, Vα14-Vβ7 and Vα24-Vβ11 NKTCrs. These structures also revealed that differences in Vβ-chain usage nevertheless impacted on CD1d-αGalCer recognition. How differing Vβ usage would impact on recognising CD1d-restricted antigens other than αGalCer was less clear. In the absence of the crystal structures of NKTCr/CD1d-iGb3 and NKTCr/CD1d-αGalDAG, we have used an approach based on functional recognition to delineate how semi-invariant NKTCrs recognise diverse ligands. The

approach involved probing a panel of naturally occurring Vα14 TCRs and derived point mutants that have altered CDRs with a panel of CD1d-restricted glycolipids and their sugar variants. Importantly, the recently solved crystal structure of the Vα14-Vβ8.2 NKTCr/CD1d-αGalCer structure enabled us to ascertain the mutations that are likely to exert indirect effects (e.g., CDR1α: V26A, P28A, H31A, L32A, R33A; CDR1β: H27A, N29A; CDR2β: S47A; G49A (Pellicci *et al*, 2009) versus mutations that would impact directly on recognition.

Figure 6 The α GalDAG recognition logic is distinct from that of α GalCer or iGb3 recognition by NKTCr. BMDs pulsed with 20 μ g/ml α GalDAG were used to stimulate a panel of NKTCr mutants and activation was measured as described in Figure 4A. Data were normalised as described in Figure 4A and represent the mean $\pm$ s.e.m. of at least three independent experiments performed in triplicate.

Our results revealed several hitherto unknown key features of diverse ligand recognition by NKTCr: first, we found that distinct epitopic sites in α GalCer and iGb3 were critical for recognition. Second, recognition of weak CD1d-restricted NKTCr agonists critically depended on the β -chain that paired with the V α 14 α -chain. Third, we discovered that while the NKTCrs dock on CD1d with a conserved footprint, they are malleable enough to recognise α GalDAG, which is structurally related to α GalCer, and the structurally most divergent iGb3 (Figure 7B). Fourth, as we will discuss below, our data best fit the 'squash' model for iGb3 recognition by the NKTCr (Figure 7A).

We found that the V α 14 NKTCr is intolerant to chemical modifications at the 3' position of α GalCer even though the 3'-hydroxyl is dispensable for NKTCr recognition (Wun *et al*, 2008). On the other hand, the V α 14 NKTCr was tolerant to multiple 4' modifications, consistent with the previous finding that the 4'-hydroxyl is dispensable for recognition (Wun *et al*, 2008). Nonetheless, a unique modification of α GalCer with 4'-N-acetyl was very poorly recognised by V α 14J α 18-V β 8.2J β 1.1 TCR. Its recognition was rescued by several mutations within CDR1 α , CDR2 α , CDR1 β and CDR3 β , many of which are surprisingly outside the common binding footprint. Thus, the CDR β loops appear to complement the recognition landscape formed by the α -chain CDRs.

The 2'''- and 3'''-hydroxyls of the terminal α 1,3Gal of iGb3 were essential for V α 14J α 18-V β 8.2J β 1.1 recognition. As with the 4'-N-acetyl- α GalCer variant, mutations within CDR1 α , CDR2 α and CDR3 β rescued recognition of both the deoxy-iGb3 variants. Because this and previous mutagenesis studies revealed germline-encoded CDR1 α , CDR3 α and CDR2 β 'hot-spots' in α GalCer and iGb3 recognition (Scott-Browne *et al*,

2007), we assume that the V α 14 NKTCr docks on both CD1d- α GalCer and CD1d-iGb3 complexes in a similar mode. The perpendicular disposition of the trihexoses of iGb3 in an unliganded state (Zajonc *et al*, 2008) as compared with the parallel orientation of the monohexose in CD1d- α GalCer (Zajonc *et al*, 2005) structure poses a serious recognition problem, and would result in steric clashes with the NKTCr (Figure 7A) if one assumed a docking mode similar with respect to CD1d- α GalCer recognition. Thus, we suggest that the glycan moiety of iGb3 is flattened upon NKTCr engagement, which is analogous to the TCR-induced flattening of extremely bulged pMHC class-I ligands (Tynan *et al*, 2007). However, with our current data, it is not possible to speculate on precisely how iGb3 might be flattened to fit into an NKTCr/CD1d-antigen complex. Notwithstanding, because LacCer and Gb3, which are related to iGb3, are not recognised by the NKTCr (Zhou *et al*, 2004), it appears as though the terminal α 1-3Gal in iGb3, in its squashed position, provides critical new contact(s) that are required for binding to CD1d, NKTCr or both.

CD1d- α GalDAG recognition by the V α 14 NKTCr also involves the previously reported germline-encoded 'hotspots' made up of residues within CDR1 α , CDR3 α and CDR2 β . In addition, it involves additional unique residues contributed by other CDR many of which are dispensable for α GalCer and iGb3 recognition. Most importantly, CDR2 α V50, K53 and D54 were critical for α GalDAG recognition. These results suggest that the positioning of the galactosyl group in α GalDAG differs from that of α GalCer. In the recently reported model, the diacylglycerol backbone of α GalDAG is predicted not to form the critical hydrogen bond network with CD1d R79 and D80. Hence, it is expected that the galactose in α GalDAG is raised above the plane of the galactose in α GalCer and is moved away from the α 2-helix (Kinjo *et al*, 2006). A similar disposition of the galactose is observed when human CD1d displays α GalCer that is caused by a species variation (human W153, which is equivalent to mouse G155; Koch *et al*, 2005; Zajonc *et al*, 2005). The putative differences in the disposition between the galactoses of α GalDAG and α GalCer when displayed by CD1d might explain the distinct logic used by the NKTCr for α GalDAG and α GalCer recognition.

We found that the β -chain played a significant role in 4'-N-acetyl- α GalCer, iGb3, iGb3 analogues and α GalDAG recognition because none of the V β 14+ NKTCr recognised these ligands. This could partly be because V β 14 lacks the CDR2 β Y48 residue, which is critically important for the binding of V β 8.2-containing NKTCr to CD1d (Borg *et al*, 2007; Scott-Browne *et al*, 2007; Wun *et al*, 2008; Mallevaey *et al*, 2009). Equally intriguing was the finding that despite the lack of Y48, V β 14+ NKTCr recognised α GalCer. This is perhaps because Y46 present in V β 14 compensates for the absence of Y48. Notwithstanding, V β 14+ NKTCr did not recognise iGb3 or α GalDAG. As well as because CDR2 β Y46-containing V β 6+ NKTCr does not recognise any of the ligands tested, we predict that CDR3 β contributes significantly to the energetic landscape for low-affinity ligand recognition (4'-N-acetyl- α GalCer, α GalDAG and iGb3), perhaps by influencing the structure of the closely disposed CDR α loops. A recent report identified an I/LxxPI/L (I, L and P are isoleucine, leucine and proline; x is any amino acid) motif within CDR3 β as a potential contributor for V β 6+ NKTCr antigen recognition (Mallevaey *et al*, 2009). Such a recognition logic would then

Figure 7 The recognition logic. (A) iGb3 modelled onto the V α 14V β 8.2/CD1d- α GalCer crystal structure shows clashes with the CDR1 α and CDR3 α loops. CDR1 α (orange); CDR2 α (green); CDR3 α (blue); CD1d (grey) and iGb3 (yellow). (B) The V α 14V β 8.2 NKTcr surface showing CDR loops (top panel). Mutations that decreased ligand binding are shown in red (middle panel). The NKTcr surface with α GalCer (left), iGb3 (middle) and α GalDAG (right). Mutations that increased ligand binding are shown in dark blue (bottom panel). The NKTcr surface with 4'-N-acetyl analogue of α GalCer (left) and 2'''-deoxy analogue of iGb3 (right). TCR surface (pale cyan); CDR1 α (orange); CDR2 α (green); CDR3 α (blue); CDR1 β (pale green); CDR2 β (pink) and CD1d helices (dark grey); CDR3 β is not shown.

explain the exclusion of the majority of the β -chains from the NKTcr repertoire during positive selection (Mallevaey *et al*, 2009), which is presumably mediated by low-affinity self-ligands. Thus, we conclude that the β -chain CDRs fine-tune the affinity threshold for NKTcr ligand recognition.

While CDR3 β did not appear to play a key role in recognition of α GalCer, variations in its length and composition did impact on the recognition of analogues of α GalCer and iGb3, as well as α GalDAG. Existing structural data (Borg *et al*, 2007; Pellicci *et al*, 2009) have not supported a key role for CDR3 β in direct recognition of CD1d- α GalCer; however, some CDR3 β conformations appear to be more permissive than others for CD1d-glycolipid-antigen recognition (Kjer-Nielsen *et al*, 2006; Scott-Browne *et al*, 2007; Mallevaey *et al*, 2009).

Taken together, the impact of CDR3 β conformation seems to vary as a function of the glycolipid antigen. It is difficult to speculate on precisely how CDR3 β is contributing to recognition in these instances, although some CDR3 β conformations might inhibit binding through steric hindrance, as suggested for the human NKT-15 TCR (Kjer-Nielsen *et al*, 2006), and some may indirectly affect recognition by altering the juxtaposition of other CDR regions that are involved in direct binding as has been observed in the structure of V α 14-V β 7 NKTcr/CD1d- α GalCer complex in comparison to that of V α 14-V β 8.2 NKTcr/CD1d- α GalCer complex (Pellicci *et al*, 2009).

In sum, the second site-suppression strategy allowed us to gain new insight into how the NKTcr recognises multiple

ligands, especially of CD1d-iGb3 and CD1d- α GalDAG complexes for which crystal structures of the receptor/antigen co-complexes are yet to be solved. Our data reinforce the previously reported common docking footprint, but extend the recognition logic significantly further for the recognition of weak/low-affinity agonists. Such weak agonists require other areas of the recognition landscape (e.g., CDR2 α , CDR1 β and CDR3 β) to achieve the energy necessary for NKT-cell activation. Therefore, we conclude that there is a consistent logic with which the NKTcr positions its 'hotspot' to find the epitope. The malleable nature of NKTcrs described herein then determines whether the orientation with which they 'perch' on the antigens, especially weak agonists, can be stabilised sufficiently to generate an activation signal. These new insights can be used to design altered glycolipid ligands that will have the potential to steer downstream immune responses in a desired manner.

Materials and methods

Mice

C57BL/6 mice were purchased from the Jackson Laboratory and used in compliance with Vanderbilt Institutional Animal Care and Use Committee-approved regulations.

Glycolipids

The syntheses and structure validation of all α GalCer and iGb3 and their variants are described elsewhere (Chen *et al*, 2007; Xia *et al*, 2009). α GalCer and iGb3 and their sugar variants were dissolved in DMSO at 1 mg/ml and used at the indicated concentrations. α GalDAG preparation and use has been reported (Kinjo *et al*, 2006).

Preparation of BMDC

BM cells were harvested from the femurs of 8 to 10-week-old C57BL/6 mice and differentiated into DC by cultivating precursors in petridishes for 7 days in RPMI + (RPMI-1640 supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1 mM penicillin/streptomycin; CellGrow) containing 20 ng/ml recombinant murine colony-stimulating factor-2 (rCsf-2; PeproTech). On days 3 and 5 of culture, 70% of the medium containing non-adherent cells was removed and replaced with fresh medium containing 20 ng/ml of rCsf-2. On day 7, the loosely adherent and non-adherent cells largely containing BMDCs and some granulocytes were harvested and used immediately for antigen loading.

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NKT hybridoma stimulation and ELISA

All Valpha14+ mouse NKT hybridomas N38-3C3, DN32.D3, N37-1H5a, N38-2C12, N57-2C12, N57-2B6, H41-2C9, H41-3C5, H41-2D9, N38-2H4, Valpha14-DO β and its alanine mutants have been described (Lantz and Bendelac, 1994; Gui *et al*, 2001; Scott-Browne *et al*, 2007). TCR α - and β -chain usage and CDR sequences are shown in Supplementary Table S1. They were maintained in RPMI + containing 55 μ M 2-mercaptoethanol. BMDCs were dispensed into round-bottomed microtitre plates at a density of 3×10^4 cells/well and incubated with increasing concentrations of α GalCer and its 3' and 4' variants, (0.1–500 ng/ml), iGb3 and its deoxy variants (10 μ g/ml) or α GalDAG (20 μ g/ml) for 18–24 h at 37°C. Lipid-loaded BMDCs were washed twice with warm PBS and co-cultivated with 6×10^4 NKT hybridoma cells per well for 24 h at 37°C. Cell-free supernatant was harvested and IL-2 secreted during co-culture was determined by ELISA.

TCR modelling

The NKTcr/CD1d-iGb3 model was prepared based on the previously reported crystal structure of CD1d-iGb3 complex (Zajonc *et al*, 2008) and the Valpha14V β 8.2 crystal structure (Pellicci *et al*, 2009). All NKTcr-CD1d models were prepared in an identical docking orientation as observed in the Valpha14V β 8.2/CD1d- α GalCer crystal structure (Pellicci *et al*, 2009).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: WCF, LEG and SJ designed experiments. WCF and LEG performed experiments. CX, WC, and YZ synthesised glycolipids; OP, LK-N and RJ provided modelled structures based on mutagenesis data; JS-B, YK, KOAY, SK, SKR, SAP, ARH, KH, and LG provided critical reagents; WCF, CX, JR, DIG, DMZ, PGW and SJ wrote and WCF, DGP, DIG, JR, LG, SAP, ARH, DMZ, PGW and SJ edited the paper.

Conflict of interest

SA Porcelli serves as a paid consultant for Vaccinex Inc., Rochester, NY. The remaining authors have no conflicting financial interests.

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