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Human Invariant NKT Cells Display Alloreactivity Instructed by Invariant TCR-CD1d Interaction and Killer Ig Receptors

Scott Patterson,* Aristeidis Chaidos,* David C. A. Neville,† Alessandro Poggi,‡ Terry D. Butters,† Irene A. G. Roberts,* and Anastasios Karadimitris2,*

Invariant NKT (iNKT) cells are a subset of highly conserved immunoregulatory T cells that modify a variety of immune responses, including alloreactivity. Central to their function is the interaction of the invariant TCR with glycosphingolipid (GSL) ligands presented by the nonpolymorphic MHC class I molecule CD1d and their ability to secrete rapidly large amounts of immunomodulatory cytokines when activated. Whether iNKT cells, like NK and conventional T cells, can directly display alloreactivity is not known. We show in this study that human iNKT cells and APC can establish a direct cross-talk leading to preferential maturation of allogeneic APC and a considerably higher reactivity of iNKT cells cultured with allogeneic rather than autologous APC. Although the allogeneic activation of iNKT cells is invariant TCR-CD1d interaction-dependent, GSL profiling suggests it does not involve the recognition of disparate CD1d/GSL complexes. Instead, we show that contrary to previous reports, iNKT cells, like NK and T cells, express killer Ig receptors at a frequency similar to that of conventional T cells and that iNKT cell allogeneic activation requires up-regulation and function of activating killer Ig receptors. Thus, iNKT cells can display alloreactivity, for which they use mechanisms characteristic of both NK and conventional T cells. The Journal of Immunology, 2008, 181: 3268–3276.

I
nvariant NKT (iNKT)3 cells are a group of regulatory T cells activated by glycosphingolipid (GSL) and phospholipid ligands presented to them by CD1d, a MHC class I-like nonpolymorphic molecule (1, 2). In evolutionary and functional terms, iNKT cells share features of T cell-dependent adaptive immunity as well as NK cell-dependent innate immunity; hence, their expression of NK cell markers and the term NKT cells (1, 2).

iNKT cells can display autoreactivity (3, 4), i.e., they can be activated by autologous CD1d-expressing APC, suggesting that they recognize endogenous ligands, the nature of which remains a matter of controversy (5). Exogenous as well as endogenous pathogen-derived α-glycuronylceramides and pollen-derived phospholipids (5) have been shown to be presented by CD1d and to activate iNKT cells. However, their most powerful, although not physiological, exogenous activating ligand is α-galactosylceramide (α-GC) (6).

Structurally, iNKT cells are characterized by a highly conserved invariant TCR α-chain (Vα24 in humans and its homologue Vα14 in mice) that pairs with a restricted set of variant Vβ-chains (preferentially with Vβ11 in humans or Vβ8 in mice) (7). iNKT cell-mediated modulation of immune responses depends on its unique ability to rapidly secrete large amounts of Th1 and Th2 cytokines (2, 3) and its ability to enhance the maturation of APC through direct interaction of the invariant TCR with CD1d expressed on APC (8–11) and possibly through interactions with Tregs (12).

Extensive work in mice has established iNKT cells as powerful modulators of immune responses against pathogens, tumors, and autoimmune and alloimmune responses (1, 13). Host iNKT cells have been shown to be required for tolerance induction in solid organ transplantation (14–16) and for the abrogation of acute graft-vs-host disease (aGVHD) in allogeneic hematopoietic stem cell transplantation (17, 18). However, donor iNKT cells appear to favor the development of aGVHD, as their removal from the graft leads to aGVHD amelioration (19). Consistent with such a role, we previously showed that human donor iNKT cells are activated in the course of in vitro alloresponses as assessed by MLR, secrete IFN-γ, and thus contribute considerably to the reactivity of the alloresponse; conversely, removal of donor iNKT cells ameliorates alloreactivity (20).

In an allogeneic setting, the activation of iNKT cells could depend directly on invariant TCR-CD1d interaction or maybe indirectly, depending on APC-derived IL-12 independently of invariant TCR and CD1d. In the former case, allogeneic activation of iNKT cells would require the invariant TCR to interact with structurally disparate CD1d/GSL complexes. However, because CD1d is an essentially monomorphic molecule (21), any structural

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3 Abbreviations used in this paper: iNKT, invariant NKT; αGC, α-galactosylceramide; a-GVHD, acute graft-vs-host disease; DC, dendritic cell; GSL, glycosphingolipid; KIR, killer Ig receptor.

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variability of the CD1d/GSL complex would be imparted by the presented GSL.

Alternatively, iNKT cells could be activated by the same molecular mechanisms responsible for the killer Ig receptor (KIR)-dependent alloactivity displayed by NK cells.

KIR are a group of polymorphic receptors that are expressed by a subset of memory T cells as well as by NK cells (22, 23). They are encoded by several different genes organized in two haplotypes of variable gene content, are inherited independently of MHC, and are expressed in a variegated manner, i.e., different combinations of KIR are expressed in different cells, but their expression remains fixed and stable in all clonally derived cells (24). Depending on their effect on NK cell reactivity upon engagement by their respective ligands, KIR are inhibitory or activating. The ligands of inhibitory KIR are polymorphic HLA class I determinants and their interaction determines the function of NK cells; in an autologous setting ligand engagement of the inhibitory KIR inhibits NK cell cytotoxic activity, whereas in an allogeneic setting the lack of HLA class I ligands results in silencing of the inhibitory receptors. activation of NK cells, and target cell lysis (“missing self” hypothesis) (25).

Some activating KIR appear to bind to the same HLA class I ligands as their inhibitory counterparts, but with a much lower activity (26, 27). In other cases, such as KIR2DS4, no binding to HLA class I ligands could be demonstrated but instead the presence of other, non-HLA class I ligands was suggested (28, 29).

The role of KIR on T cells is less clear, with some evidence suggesting that they may inhibit TCR-mediated T cell activation while other data suggest they may function as costimulatory molecules facilitating T cell activation (30).

In this work we investigate whether human iNKT cells can be activated in an allogeneic manner through a direct cross-talk with APC and also dissect the molecular requirements and especially the potential role of KIR in this process.

Materials and Methods

Cells and cell culture

PBMC were obtained after Ficoll fractionation of normal blood donor buffy coat preparations provided by the North London Blood Transfusion Centre (London, U.K.) under local Research Ethics Committee approval. The CD14 positive selection kit (StemCell Technologies) was used as per the manufacturer’s instructions to select peripheral blood monocytes. Selected cells were >90% CD14+.

In vitro expansion of iNKT cells in the presence of aGC was performed as previously described (31).

Monoclonal Abs, flow cytometry, and flow sorting

Cells were stained with mAbs using standard protocols. The anti-KIR clones used (either purified or as supernatants) have been described previously (32, 33) and were as follows: the pan-anti-KIR2D (CD158abhi) clone NKVFS1, anti-CD158b clone GL183, anti-CD158e clone Z27, and anti-CD158i clone PES172. The following mouse anti-human mAbs were also used: FITC-, R-phycocerythrin (RPE)-, or biotin-labeled anti-TCRVα24 and TCRβ11 (Serotec; Beckman Coulter); CD3-allophycocyanin, IgG1-FITC, IgG1-RPE, and IgG1-biotin (Caltag Laboratories); CD3-PerCP, HLA-DR-allophycocyanin, streptavidin-allophycocyanin, and PE-labeled anti-invariant TCRVα18 chain clone 6B11 (BD Biosciences). CD86, CD14, CD83, and CD1d/aGC tetramer were provided by S. Gadola, University of Southampton, Southampton, U.K. Multicolor flow cytometry was performed using a FACSCalibur, whereas flow sorting was performed using a FACSaria (BD Biosciences). Data analysis was performed with FlowJo software.

Generation of iNKT cell clones

For the generation of iNKT cell clones, PBMC from two different donors were first stained with PE-labeled anti-TCRVα24 followed by positive immunomagnetic anti-PE bead selection. TCRVα24-enriched cells were subsequently cloned at 1 cell/well to a 96-well plate by flow sorting (FACSaria) in the presence of third party PBMC as feeder cells at 50 × 10^4/well and IL-2 at 50 U/ml every 3 days. Cells were also stimulated with PHA at 1 μg/ml on day 1 and then every 2 wk. Invariant NKT cells were identified as TCRVα24+ TCRβ11+ or CD1d/aGC tetramer “TCRVα24+” or invariant TCRVα24+ TCRβ18+ TCRVα24+ cells. For functional assays, iNKT cells rested (i.e., without PHA stimulation) for at least 2 wk were used.

Invariant NKT cell MLR and proliferation assays

For all cultures, T cell medium consisting of RPMI 1640 and 5% heat inactivated AB human serum supplemented with 1% l-glutamine and penicillin/streptomycin was used. For iNKT MLR, generally 2–3 × 10^7 freshly flow-sorted, 10–20 × 10^4 aGC-expanded, or 30–50 × 10^3 clonal iNKT cells were placed in triplicate against autologous or allogeneic irradiated (3000 rad) monocytes in 96-well plates at responder:stimulator ratios as indicated in the text. T lymphotimidine was added at 1 μCi/well for the last 16 h of the MLR. Proliferation was measured using a liquid scintillation counter after harvesting with a cell harvester. The following mAbs were used for blocking experiments: IgG1 isotype and anti-CD1d clone 42.1 (both from BD Pharmingen), anti-TCRVα24, and anti-pan-KIR2D (clone NKVFS1), all at 10 μg/ml.

Monocyte maturation assay

Purified monocytes (2 × 10^5) were cocultured with iNKT cell clones (4 × 10^6). As controls, monocytes were treated with T cell medium only or with a combination of GM-CSF (50 ng/ml) and IL-4 (1000 U/ml). After 4 days, expression of CD83, CD86, CD14, and HLA-DR was performed by flow cytometry. Monocytes were identified by their physical characteristics and lack of expression of CD3.

Redirected cell lysis assay

For the redirected cell lysis assay, iNKT cell clones that had been preactivated for 6–8 h either with autologous or allogeneic monocytes were placed against the FcγRIIb+ P815 marine mastocytoma cell line in the presence of 10 μg/ml anti-CD158ab (clone NKVFS1) or anti-CD3 (1 μg/ml; clone UCHT1; BD Pharmingen) mAbs using the Cytotox96 nonradioactive cytotoxicity kit (Promega) according to the manufacturer’s instructions. All assays were performed in triplicate. Specific target cell lysis was calculated according to the following formula: percentage of cytotoxicity = (experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous) × 100.

RT-PCR for KIR gene expression

Total RNA was isolated using TRIzol (Invitrogen). After DNase I treatment, first strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen).

The hybridization specificity of the primers used for KIR cDNA amplification was checked against the GenBank and European Molecular Biology Laboratory databases. PCR conditions were as follows: initial denaturation at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min. The sequence of the cDNA-specific primers were as follows: for KIR2DL4 (CD158d), 5‘-TCTATGGTACCTCCTCA CGAG-3’ (forward) and 5‘-AAGAGGATGAGTGCCACCTG-3’ (reverse); for KIR3DL2 (CD158k), 5‘-GCCCACAGTCAACACAGAACAT CAT-3’ (forward) and 5‘-ACCATGAGTGGCCATGCAATCG-3’ (reverse); and for KIR2DL1 (CD158a), 5‘-CGGCTTCTTCCATGACTCTC-3’ (forward) and 5‘-GGCTCTTCCATGACTCTC-3’ (reverse).

GSL analysis

GSL analysis was performed on peripheral blood B cells purified using the immunomagnetic bead CD19 positive selection kit (Miltenyi Biotec) and mature DC prepared as previously described (31). GSL extraction was performed essentially as described (34).

ELISAs

Quantiﬁcation of IFN-γ and IL-12p70 in the supernatants of the iNKT cell-monocyte MLR was performed with the Quantikine kit (R&D Systems).

Results

Alloreactive activation of iNKT cells

We tested whether iNKT cells would be preferentially activated by allogeneic rather than autologous APC. For this purpose we generated CD1d/aGC-tetramer+ iNKT cell clones from two
normal donors (NKT cells clones 37 and 49; Fig. 1A), and in proliferation assays we tested their reactivity against autologous or allogeneic monocytes as APC (Fig. 1B). We found that in the presence of allogeneic APC, proliferation of iNKT cell clone 49 was, in a dose- and time-dependent-manner, considerably higher than in the presence of autologous APC and was substantially inhibited by anti-CD1d and anti-TCRVα24 mAbs (Fig. 1B). In addition, highly purified (>98%; data not shown) aGC-expanded iNKT cell lines (Fig. 1D) as well as ex vivo purified (>98%) iNKT cells (Fig. 1C) from donor 49 proliferated in a similar alloreactive and CD1d-dependent manner. Similar results were obtained using clones, ex vivo purified or aGC-expanded iNKT cells from another four donors (data not shown). These results establish that iNKT cells can be activated in an allogeneic, CD1d-dependent, and invariant TCR-dependent manner.

iNKT cells preferentially induce differentiation of monocytes toward dendritic cells (DC)

Having established that allogeneic APC are much more effective in activating iNKT cells as compared with autologous APC, we next investigated whether this effect is associated with enhanced and preferential induction of APC maturation by allogeneic rather than autologous iNKT cells. In coculture experiments we found that differentiation of monocytes toward mature myeloid DC, as determined by surface expression of costimulatory molecules, cytokine secretion, and functional assays, was more effectively induced when they were cocultured with allogeneic rather than autologous iNKT cells. Specifically, expression of CD86 and HLA-DR (but not CD83) was considerably higher in monocytes cocultured with the allogeneic clone 37 as opposed to autologous iNKT cell clone 49 and comparable to that of monocytes treated with GM-CSF/IL-4; this was accompanied by significant down-regulation of CD14 expression in the allogeneic but not autologous setting (Fig. 2A). In addition, considerably higher levels of IL-12 (Fig. 2B), a Th1 cytokine secreted by mature myeloid DC, was detected in the alloreactive setting as compared with the autologous setting, a finding associated with higher secretion of IFN-γ by allogeneic rather than autologous iNKT cells (Fig. 2C). The enhanced myeloid DC maturation effect of alloreactive iNKT cells was also evident in the third party MLR assays in which the DC that matured in the presence of allogeneic iNKT cells were much more efficient in eliciting an alloresponse by third party T cells as compared with DC matured in the presence of autologous iNKT cells (Fig. 2D). Taken together, the findings in Figs. 1 and 2 suggest that in an allogeneic setting iNKT cells and DC are able to establish an exclusive cross-talk that involves invariant TCR-CD1d interactions and a cytokine circuit of IL-12 and IFN-γ secretion that is sufficient to lead to their activation and maturation, respectively.

GSL profiling of APC and iNKT cell alloreactivity

Because the allogeneic function of iNKT cells is invariant TCR-CD1d interaction dependent, it could be argued that in this interaction and in a manner similar to that of conventional T cell-driven allosresponses whereby the allogeneic TCR interacts with disparate HLA class I or II/peptide complexes, invariant TCR reacts to disparate CD1d/GSL complexes. Therefore, in principle, iNKT cell alloreactivity could be driven either by structural variations in CD1d or in the presented GSL. The first possibility can be ruled out, as it is well established that CD1d is essentially monomorphic (21). To address the second possibility, using a highly sensitive and specific HPLC-based method for GSL analysis (34) we compared the GSL profile of mature DC and B cells from eight normal individuals. Although the GSL profiles of DC and B cells were different, we found no consistent differences in the polar and non-polar GSL profiles among different normal donors (Table I), suggesting that structural GSL variation is unlikely to underlie the preferential activation of iNKT cells by allogeneic APC and that
although the CD1d-invariant TCR interaction is required for iNKT cell alloreactivity, it is not the cause of it.

Expression of KIR in human iNKT cells
We considered the possibility that alloactivation of iNKT cells might resemble that of NK rather than that of conventional T cells; i.e., involve KIR. Indeed, human as well as murine iNKT cells have been shown to express various NK cell receptors including CD161 and CD94/NKG2 (35, 36). However, although murine iNKT cells express Ly49 molecules, the murine equivalents of KIR, these have been reported not to be expressed in human iNKT cells (36, 37).

To investigate this discrepancy, we studied KIR expression in ex vivo identified (Fig. 3A) or in vitro aGC-expanded iNKT cells (Fig. 3B), as well as in the iNKT cell clones described above (Fig. 3C). Using flow cytometry and staining with KIR-specific mAbs, we found that polyclonal ex vivo or in vitro aGC-expanded iNKT cells do express KIR but at low frequencies, comparable to those of conventional T cells, and, in accordance with the expression pattern of KIR in NK and conventional T cells, KIR expression in iNKT cells is variegated (data not shown); by contrast, in the two resting iNKT cell clones used in these experiments, expression of KIR was uniform and detectable above the isotypic controls but much lower compared with that in NK and T cells (Fig. 3C and data not shown).

Expression of KIR by iNKT cell clones was also shown by RT-PCR (Fig. 4A). Using primers specific for cDNA amplification, we found expressions of CD158d (KIRDL4) and CD158k (KIR3DL2), two framework (i.e., universally expressed) KIR genes (24), at the mRNA level (Fig. 3D), as well as the expression of CD158a (KIR2DL1); further, immunoblotting using the antianpan-KIR mAb clone NKVFS1 showed expression in the iNKT cell clones but not in monocytes (Fig. 4B).

KIR-dependent alloreactivity of iNKT cells
Having established that KIR are expressed on human iNKT cells, we investigated their role in the iNKT cell-based alloresponse. First, the dynamics of KIR expression on the...
iNKT cells during the course of the MLR was assessed. For this purpose, resting iNKT cell clone 49 was placed in coculture with either autologous or allogeneic monocytes and, using the anti-pan-KIR2D mAb clone NKVFS1, KIR expression was studied at two different time points. We observed that iNKT cells activated in the allogeneic rather than autologous MLR

FIGURE 3. Expression of KIR in iNKT cells by flow cytometry. A, KIR expression in ex vivo identified iNKT cells (CD3⁺TCRVα24⁺Vβ11⁺) as compared with NK (CD3⁺CD56⁺) and conventional T cells (CD3⁺) as assessed by flow-cytometry. Data are representative of three normal donors. B, KIR expression in aGC-expanded iNKT cells as compared with conventional CD3⁺ T cells. CD3⁺CD56⁻ cells are not shown because only few were present on day 14 of the culture. In this experiment, iNKT cells are stained with the invariant TCR Vα24Jα18 chain-specific mAb 6B11. Data are representative of three normal donors. C, Expression of different KIR Ags on resting iNKT cell clone 49 (gray, isotype; bold, anti-KIR staining).
expressed 5- to 6-fold higher levels of KIR, with the highest expression observed at 6 h, declining toward baseline levels at 24 h (Fig. 5[A]).

To assess the functional role of KIR in allogeneic iNKT cell activation, the reactivity of iNKT cells cocultured with autologous or allogeneic monocytes was studied in the presence of anti-CD1d, anti-pan-KIR2D mAb, or their combination. Alloreactivity, as assessed by proliferation (Fig. 5B), was inhibited by at least 50% in the presence of the anti-KIR mAb, comparable to the inhibiting effect of anti-CD1d, and by 75% when anti-KIR was combined with anti-CD1d. In addition, iNKT cells were considerably more cytotoxic against allogeneic than autologous monocytes, an effect abrogated in the presence of the anti-pan-KIR2D mAb (Fig. 5C).

Because clone NKVFS1, the anti-KIR mAb used in these assays, can recognize inhibiting as well as activating isoforms of KIR2D with the same affinity and avidity, the observed effect on NK cell activation is (usually) the opposite of that obtained when KIR are engaged by their natural ligands, i.e., engagement of inhibitory KIR by their ligands results in NK cell inhibition, whereas NK cells are activated when activatory KIR are engaged by their respective ligands. Therefore, the inhibiting effect of this mAb, besides indicating the requirement for KIR in the alloreactive activation of iNKT cells, is also likely to reflect the prevalence of KIR-associated activating signals over inhibitory ones.

To confirm that during the course of the allogeneic activation of iNKT cells activating KIR indeed prevail over inhibiting ones, we used a re-directed cell lysis assay (33, 38) in which the anti-pan-KIR2D mAb was used to cross-link KIR on iNKT cells with the Fc receptors of the P815 mastocytoma cells (Fig. 5D). We found that in the presence of anti-KIR, the lytic ability of iNKT cells preactivated for 6–8 h in coculture with allogeneic monocytes was considerably higher than that of iNKT cells cocultured with autologous monocytes. At the same time, the levels of CD3-based redirected lysis imparted by iNKT cells activated by autologous and allogeneic APC were comparable, indicating that iNKT cells cocultured in the autologous setting do not have an intrinsic cytotoxicity defect. Taken together, these findings suggest that preferential induction and activation of activating KIR is important for the allogeneic activation of iNKT cells.

FIGURE 4. Expression of KIR in iNKT cells by RT-PCR and immunoblotting. A, Expression by RT-PCR of CD158d (KIRDL4) and CD158k (KIR3DL2), two framework KIR genes, and CD158a (KIR2DL1) in iNKT cell clones 37 and 49, peripheral blood mononuclear blood cells, and highly purified monocytes is shown. G6PD expression is shown as an internal control. Sizes of the specific PCR products are also shown. B, Lysates from iNKT cell clones, PBMC, purified monocytes, and PBMC without the selected monocytes were subjected to SDS-PAGE and immunoblotting using the anti-CD158 pan-KIR2D or anti-actin mAb. Strong expression of KIR is detected in PBMC, the iNKT cell clones 37 and 49, and PBMC minus monocytes; in contrast, only a weak band appears in the monocyte lane, probably representing slight contamination with T and NK cells following CD14 immunomagnetic bead positive selection of PBMC.

FIGURE 5. Role of KIR in iNKT cell alloreactivity. A, Dynamic expression of KIR in allogeneically activated iNKT cells as assessed by flow cytometry. Staining with the anti-CD158abhi (pan-KIR2D) mAb of iNKT clone 49 at baseline and after 6 and 24 h of coculture with autologous or allogeneic monocytes. B, Proliferation of iNKT clone 49 against allogeneic or autologous monocytes in the presence of the indicated mAb. C, Cytotoxicity of iNKT cell clone 49 against allogeneic or autologous monocytes and the effect of the anti-pan-KIR2D mAb. Allo, Allogeneic monocytes, auto, autologous monocytes. D, Testing activating vs inhibitory KIR in redirected cytotoxicity assays. iNKT cell clone 49 was stimulated with either autologous (auto) or allogeneic monocytes (allo) for 6–8 h, and subsequently its cytotoxicity against the mastocytoma P815 cells in the presence of anti-CD3 or anti-panKIR2D was tested at the E:T cell ratios indicated. Each of the above is representative of at least three independent experiments.
**Discussion**

iNKT cells are considered as immunomodulatory cells that share many features with both NK and T cells, thus functioning as a bridge between innate and adaptive immunity. Alloreactivity is a property displayed by both NK and conventional T cells, but is not known whether it is also a feature of iNKT cells. In this study we show that iNKT cells can be also activated in an alloreactive manner, a process that involves a direct cross-talk of iNKT cells with APC. Indeed, freshly isolated or in vitro expanded iNKT cells proliferate preferentially in response to allogeneic rather than autologous monocytes. It is very unlikely that the observed effect reflects the proliferation of residual conventional allogeneic T cells, because the purity of the sorted populations was >98% (data not shown); furthermore, iNKT cell clones proliferated highly and preferentially in the presence of allogeneic monocytes in a CD1d- and invariant TCR-dependent manner.

Previous work has demonstrated the ability of iNKT cells to enhance the maturation of APC in a CD1d-dependent manner in mice as well as in humans (8–11); however, there has been no direct comparison of the effect on APC maturation by autologous or allogeneic iNKT cells. In this study, using markers of DC differentiation, the ability to secrete IL-12 and incite third party MLR, we show a much greater effect of allogeneic iNKT cells in inducing differentiation of monocytes toward DC. Therefore, iNKT cells interacting with allogeneic APC can establish a cross-talk, inducing each other’s maturation and activation leading to enhancement of the alloresponse. The finding that maximum expression of KIR (see below) occurs as early as within 6 h of the iNKT cell-monocyte coculture suggests that activation of iNKT cells probably precedes commencement of the maturation of allogeneic monocytes toward DC. Consistent with these findings, donor splenocytes from iNKT cell-deficient Jα18−/− mice were shown to induce less severe experimental aGVHD than WT donors (19), although in another study there was no difference on the severity of aGVHD but just in the levels of Th1 cytokines such as TNF-α (39). The findings presented here are also in line with our previous work showing that human iNKT cells contribute as much as 50% to the in vitro alloreactivity as assessed by conventional T cell MLR (20).

Similarly as for conventional T cells, in which alloreactivity requires TCR recognition of a disparate MHC/peptide complex, we show that allogeneic activation of iNKT cells requires direct interaction of CD1d with the invariant TCR. However, because CD1d is essentially monomorphic (21), allogeneic activation of the invariant TCR would require structural variability of the presented endogenous GSL. Using a highly sensitive approach for GSL profiling, we found no such variability among different normal individuals, making it unlikely that this is the basis of allore cognition. Thus, our findings suggest that CD1d-invariant TCR interaction, although not the cause of iNKT cell alloreactivity, it is still required for its development; it is likely that in this context, GSL presentation by CD1d is also required for the successful interaction between iNKT cells and APC that, in the absence of inhibitory signals (i.e., autologous MHC), results in alloreactivity.

We considered the possibility of KIR being involved in the process of iNKT cell alloreactivity; although it was previously reported that iNKT cells do not express KIR (36, 37), we found that similarly as in conventional CD3+ T cells, a small proportion of iNKT cells, whether ex vivo identified or in vitro expanded with aGC, clearly express KIR. It is possible that this low frequency of KIR+ iNKT cells was missed in previous studies either because insufficient numbers (>1500 in our case) were acquired for analysis (36) or because only a small number of iNKT cell clones was studied (37). In addition, the use of the anti-panKIR2D clone NKVFS1 in our study, but not in the other studies, increased significantly the ability to detect KIR+ iNKT cells by flow cytometry.

Nevertheless, our data show that upon iNKT expansion with aGC, KIR expression by iNKT cells is readily detectable. Expression of KIR was also detected in a uniform manner on the two iNKT cell clones used in our experiments, but at levels much lower than on conventional T or NK cells and was confirmed by RT-PCR and immunoblotting. The finding of KIR expression by human iNKT cells is in line with the expression by murine iNKT cells of Ly49, the KIR equivalent molecules in mice (40). However, unlike in humans, the frequency of Ly49+ iNKT cells is several fold higher than that in T cells (41).

Our next set of experiments demonstrated that allogeneic activation of iNKT cells is associated with the up-regulation of KIR in the first 6 h and the subsequent down-regulation toward baseline levels, showing that KIR expression in iNKT cells is a dynamic process. In this regard, iNKT cells are similar to T cells, which have been shown to modulate expression levels of their KIR in a MHC/peptide-dependent manner, but are dissimilar to NK cells, in which KIR expression levels remain fixed (25, 42). Previous work has shown that modulation of KIR expression in T cells involves transcriptional as well as posttranscriptional mechanisms (43, 44). Interestingly, Vely et al. have shown that KIR mRNA could be readily detected in NK cells that were negative by flow cytometric analysis of the corresponding surface KIR molecules (44). Whether the same applies for iNKT cells remains to be determined.

What might be the significance of KIR expression by iNKT cells? These cells have been shown to be autoreactive in vitro; therefore, engagement of inhibitory KIR by self-HLA class I molecules might be important to prevent iNKT cells from exerting autoreactivity in vivo under steady-state conditions. In the course of an immune response, as our data show, expression of KIR on iNKT cells can be modulated rapidly, such that the reactivity of iNKT cell is enhanced either by down-regulation of inhibitory or up-regulation of activating KIR. It is also likely that the observed ability of in vitro expanded human iNKT cells to kill tumor cell lines in vitro in a CD1d-independent manner is KIR dependent (45).

Because clone NKVFS1, the mAb used in our assays, binds with equal affinity to most if not all KIR2D isoforms (32, 33, 46), the enhanced and dynamic expression of KIR in the allogeneic setting could reflect up-regulation of either inhibitory or activating KIR. The functional assays used in the presence of the anti-KIR mAb suggested very strongly that, during the course of the iNKT cell MLR, in functional terms the activating KIR prevailed over inhibiting KIR and are very important determinants in the allogeneic activation of iNKT cells. This differs from the main mechanism of NK cell alloactivation, which involves recognition of the “missing self” as a result of the lack of inhibitory KIR ligands. Ligands for some activating KIR have not been identified to date. Earlier work suggested that activating KIR2D can bind to the same HLA-C ligands as their inhibitory KIR2D counterparts, albeit with a much lower affinity (26, 27); other lines of evidence suggest that activating KIR might bind to yet to be identified non-HLA ligands (28, 29). We found that the reactivity of iNKT cells in response to allogeneic APC in the presence of an anti-HLA class I mAb was inhibited by <25% (data not shown), implying that if HLA class I ligands were involved their interaction with activating KIR would be of low affinity, thus suggesting the presence of other,
possibly non-HLA class I ligands. The observed robust alloactivation of iNKT cells from different individuals against several stimulators would argue that these ligands could be highly polymorphic molecules.

In addition to KIR and as suggested by the finding that anti-KIR mAb did not inhibit proliferation completely but by ~50%, other NK cell receptors might also be important for allogeneic activation of iNKT cells. A good candidate fulfilling this role would be the heterodimeric NKG2A/CD94 molecule, which has been shown to be expressed in iNKT cells (35, 36). The nonconventional and nonpolymorphic HLA-E molecule, presenting peptides derived from the leader part of conventional HLA class I molecules, is the ligand of NKG2A (22, 47). Because NKG2A functions as an inhibitory receptor when engaged by HLA-E on autologous cells, it is possible that during the allogeneic stimulation of iNKT cells NKG2A does not bind to HLA-E but the latter presents peptides from disparate HLA class I molecules, leading thus to iNKT cell activation on the basis of “missing self.” In support of this, in preliminary experiments we found that allogeneic activation of iNKT cells can be partially inhibited by an anti-CD94 mAb (data not shown).

In conclusion, we have shown that similarly as NK and conventional T cells, iNKT cells can be activated in an alloreactive manner. Having established that iNKT cells express KIR, we showed that their allogeneic activation requires invariant TCR-CD1d interaction; however, allore cognition also requires the dynamic modulation of activating KIR and their engagement by yet to be identified ligands. It is reasonable therefore, based on the current and previously published data examining the role of donor iNKT cells in the induction of alloreactivity and aGVHD (19, 20), to postulate that iNKT cell depletion of the hematopoietic graft could be a means of reducing the incidence and severity of clinical aGVHD.

Disclosures
The authors have no financial conflict of interest.

References


