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*J Immunol* 2005; 175:5087-5094; doi: 10.4049/jimmunol.175.8.5087
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Human Invariant NKT Cells Are Required for Effective In Vitro Alloresponses

Scott Patterson,* Ioannis Kotsianidis,∗ Antonio Almeida,∗ Marianna Politou,* Amin Rahemtulla,∗ Bini Matthew,† Richard R. Schmidt,† Vincenzo Cerundolo,‡ Irene A. G. Roberts,* and Anastasios Karadimitris2∗

NKT cells are a small subset of regulatory T cells conserved in humans and mice. In humans they express the Vα24Jα18 invariant chain (hence invariant NKT (iNKT) cells) and are restricted by the glycolipid-presenting molecule CD1d. In mice, iNKT cells may enhance or inhibit anti-infectious and antitumor T cell responses but suppress autoimmune and alloreactive responses. We postulated that iNKT cells might also modulate human alloreactive responses. Using MLR assays we demonstrate that in the presence of the CD1d-presented glycolipid α-galactosylceramide (αGC) alloreactivity is enhanced (37 ± 12%; p < 0.001) in an iNKT cell-dependent manner. iNKT cells are activated early during the course of the MLR, presumably by natural ligands. In MLR performed without exogenous ligands, depletion of iNKT cells significantly diminished the alloresponse in terms of proliferation (58.8 ± 24%; p < 0.001) and IFN-γ secretion (43.2 ± 15.2%; p < 0.001). Importantly, adding back fresh iNKT cells restored the reactivity of iNKT cell-depleted MLR to near baseline levels. CD1d-blocking mAbs equally reduced the reactivity of the iNKT cell-recorpt and -depleted MLR compared with IgG control, indicating that the effect of iNKT cells in the in vitro alloresponse is CD1d-dependent. These findings suggest that human iNKT cells, although not essential for its development, can enhance the alloreactive response. The Journal of Immunology, 2005, 175: 5087–5094.

Received for publication April 28, 2005. Accepted for publication August 4, 2005.

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1 This work is supported by Cancer Research U.K. Grant C399-A2291 and the Cancer Research Institute (to V.C.). A.K. is a Leukaemia Research Fund Bennett Senior Fellow.

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3 Abbreviations used in this paper: iNKT, invariant NKT; αGC, α-galactosylceramide; DC, dendritic cell; GVHD, graft-vs-host disease.

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CD3+ cells, the EasySep negative selection kit was used as per manufacturer’s instructions (StemCell Technologies). Purity of CD3+ cells was always >90% (data not shown).

Flow cytometry and flow sorting

Cells were stained with mAbs using standard protocols (18). For the identification and flow sorting of INKT cells, selected CD3+ cells were stained with mAb against TCR V24 and Vβ11. With this approach the vast majority but not all bona fide INKT cells are identified (18, 19). Therefore, depletion of INKT cells using TCR V24 and Vβ11 mAbs will remove most CD1d/αGC tetramer-positive INKT cells except a very small population of CD1d/αGC tetramer-negative TCR V24-negative INKT cells (19). The following mAbs were used: mouse anti-human TCR V24 and TCR Vβ11 either FITC- or RPE-labeled, or biotin-labeled (Serotec); mouse anti-human CD69-FITC, CD3-allophycocyanin, IgG1-FITC, IgG1-RPE, and IgG1-biotin (Caltag Laboratories); and CD4-PerCP, HLA-DR-allophycocyanin, and steptavidin-allophycocyanin (BD Biosciences). Multicolor flow cytometry was performed using a FACSCalibur, whereas flow sorting was performed using a FACS Diva (BD Biosciences). Data analysis was performed with the CellPro or FlowJo software.

MLR and proliferation assays

For all cultures, T cell medium consisting of RPMI 1640, 5% heat-inactivated human serum supplemented with 1% L-glutamine and penicillin/streptomycin was used. For MLR, generally 30–50 × 10^3 CD3+ cells were placed in triplicates against autologous or allogeneic irradiated (3000 rad) PBMC in 96-well plates at responder to stimulator (R:S) ratios as indicated. For long-term MLR, 30–50 × 10^4 CD3+ cells were plated in 48-well plates. [3H]Thymidine 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. Parallel cultures were set for cytokine release assays. In indicated experiments, αGC (100 ng/ml) or its diluent vehicle (NaCl 150 mM/Tween 20 0.05%) were added to the MLR. The following mAbs were used for blocking MLR: anti-CD1d (clone 42.1; BD Biosciences).

**FIGURE 1.** αGC enhances alloreactivity in an INKT cell-dependent manner. a, MLR was performed using T cells from responder 86 against a panel of five stimulators in the presence of vehicle or αGC (top). In the presence of αGC, proliferation increased by 48% (range from 25 to 73%). Minimal reactivity was observed in autologous MLR. In total, MLR of four responders against five stimulators (each repeated twice) were tested and in all cases addition of αGC (bottom) had an enhancing effect on the alloreactive response (37 ± 12%; p < 0.001). b, IFN-γ production was increased in αGC-treated allogenic compared with vehicle-treated MLR but not in autologous MLR. No IL-4 production was detected under these conditions. Data are representative of four independent MLR. c, INKT cell-replete or cell-depleted MLR were performed in the presence of vehicle or αGC. In INKT cell-depleted allo-MLR, proliferation was equally reduced under both conditions, indicating that the enhancing effect of αGC on the alloresponse is INKT cell-dependent (CD3+ indicates INKT cell-replete MLR, and CD3+ NKT− indicates INKT cell-depleted MLR). d, Proliferation as determined by CFSE staining (upper) and absolute numbers (middle) of INKT cells (as determined by flow cytometry and total cell count) in a 96-h MLR in the presence of αGC or vehicle. In parallel, proliferation was measured by [3H] incorporation (lower). Blots are gated on INKT cells identified by anti-TCR V24 and Vβ11 staining. INKT cells proliferate more in the presence of αGC; however, their increase in absolute numbers does not account for the 50% increase in proliferation observed in the presence of αGC. Data are representative of two independent experiments and are presented as mean ± SEM of triplicate assays.
Pharmingen); anti-HLA class II (clone TU39; BD Pharmingen); and IgG1 isotype (BD Pharmingen) at indicated concentrations.

CFSE (Molecular Probes) staining of CD3<sup>+</sup>/H11001 cells was performed in PBS for 8 min at room temperature using CFSE at 5 μM with occasional mixing. After washing with an equal volume of FCS, cells were extensively washed with complete T cell media.

ELISA
Quantitation of IFN-γ and IL-4 in the supernatants of the MLR was performed with the Quantikine kit (R&D Systems).

Statistical analysis
The Wilcoxon signed ranks test was used to compare differences in proliferation between iNKT cell-replete and cell-depleted MLR and between αGC or vehicle-treated MLR.

Results
Invariant NKT cell-dependent enhancement of MLR by αGC

In in vivo murine models, treatment of irradiated recipient mice with αGC induces host-derived, iNKT cell-dependent IL-4 secretion and protection from acute GVHD (11, 12). We tested the effect of αGC in human in vitro alloresponses. For this purpose we performed 4-day MLR, using as responders purified negatively selected T cells and as stimulators irradiated PBMC pulsed with αGC or vehicle. As shown in Fig. 1a, top, addition of αGC to the MLR of responder 86 against a panel of five stimulators caused a 25–73% increase in proliferation. To rule out nonspecific T cell activation that may be induced by αGC (20), autologous MLR was performed in parallel with the allogeneic MLR. In the presence of either αGC or vehicle, minimal proliferation was observed in autologous as compared with allogeneic MLR (Fig. 1a, top), suggesting thus that the enhancing effect of αGC in the allogeneic MLR is alloreactive T cell-specific. In addition to responder 86, we tested another three responders against a panel of five stimulators and found that treatment of the MLR with αGC resulted in a 37 ± 12% (p < 0.001) increase in proliferation as compared with MLR treated with vehicle only (Fig. 1a, bottom). Consistent with this, IFN-γ production was also comparably increased in the MLR performed in the presence of αGC; no IL-4 was detected at 96 h (Fig. 1b) or at 4 and 24 h of the MLR (data not shown). Furthermore, the enhancing effect of αGC was iNKT cell-dependent, as upon iNKT cell depletion (see below) and in the presence of αGC, proliferation was equally affected as in iNKT cell-depleted MLR treated with vehicle (Fig. 1c).

To further elucidate the relative role of iNKT cells in the enhancement of MLR by αGC, we correlated proliferation of iNKT cells with αGC or vehicle. As shown in Fig. 1a, top, addition of αGC to the MLR of responder 86 against a panel of five stimulators caused a 25–73% increase in proliferation. To rule out nonspecific T cell activation that may be induced by αGC (20), autologous MLR was performed in parallel with the allogeneic MLR. In the presence of either αGC or vehicle, minimal proliferation was observed in autologous as compared with allogeneic MLR (Fig. 1a, top), suggesting thus that the enhancing effect of αGC in the allogeneic MLR is alloreactive T cell-specific. In addition to responder 86, we tested another three responders against a panel of five stimulators and found that treatment of the MLR with αGC resulted in a 37 ± 12% (p < 0.001) increase in proliferation as compared with MLR treated with vehicle only (Fig. 1a, bottom). Consistent with this, IFN-γ production was also comparably increased in the MLR performed in the presence of αGC; no IL-4 was detected at 96 h (Fig. 1b) or at 4 and 24 h of the MLR (data not shown). Furthermore, the enhancing effect of αGC was iNKT cell-dependent, as upon iNKT cell depletion (see below) and in the presence of αGC, proliferation was equally affected as in iNKT cell-depleted MLR treated with vehicle (Fig. 1c).

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FIGURE 2. Expansion and activation of iNKT cells in long-term MLR. a, Three independent MLR were performed in the presence of αGC or vehicle without exogenous IL-2 supplementation. On indicated time points the frequency as well as the activation of iNKT cells was determined by flow cytometry after staining with anti-TCR V<sup>24</sup>, V<sup>811</sup>, and CD69. At least 5 × 10<sup>4</sup> events were collected for analysis. The frequency of iNKT cells in the MLR treated with vehicle (top, solid line) remained similar to baseline throughout the course of the MLR. In the presence of αGC (dashed line), the frequency of iNKT cells on day 4 was similar to baseline; however, it increased by at least 10-fold by day 8. The frequency of naturally activated iNKT cells progressively increases during the course of the MLR (bottom). Greater activation of iNKT cells is seen when cells are pharmacologically activated by exogenous αGC. b, IFN-γ production by iNKT cells and non-iNKT T cells in MLR treated with αGC or vehicle on day 8. αGC induces more IFN-γ production by total T cells as well as iNKT cells. In addition, activation of non-iNKT cells as assessed by forward scatter characteristics is also higher in the presence of αGC. Gates for iNKT cells blots have been set on TCR V<sup>24</sup>/V<sup>811</sup> events. Gates for total CD3 cells for both blots and histograms exclude iNKT cells.
cells (as assessed by CFSE labeling) and absolute counts (as determined by flow cytometry and total cell count) with the proliferation of all T cells (as assessed by \(^{3}H\) incorporation). CFSE staining showed that iNKT cells proliferated more in the presence of \(\alpha\)GC compared with vehicle (Fig. 1d, upper). This proliferation corresponded to an absolute increase from a baseline of 300 iNKT cells on day 0 (corresponding to \(2 \times 10^6\) plated T cells) to 450 and 690 cells 4 days later in the presence of vehicle and \(\alpha\)GC, respectively (Fig. 1d, middle). At the same time, the MLR reactivity of total T cells (5 \(\times 10^4\) responder T cells corresponding to \(10^{-3}\) iNKT cells) increased by almost 50% (corresponding to \(10^4\) cpm) in the presence of \(\alpha\)GC (Fig. 1d, lower). It is very unlikely that this difference is the result of proliferation of the few NKT cells activated on day 4 by \(\alpha\)GC; instead, it reflects the proliferation of the alloreactive T cells.

**Dynamics of iNKT cell activation in MLR**

\(\alpha\)GC is a pharmacological agent and its effect on iNKT cell expansion and activation may not reflect the mechanisms of iNKT cell activation by natural CD1d ligands. The dynamics of iNKT cell expansion and activation (as determined by the surface activation marker CD69) in response to natural ligands were monitored during the course of long-term MLR (Fig. 2). In the presence of \(\alpha\)GC the relative frequency of iNKT cells did not increase significantly on day 4, but it did increase by at least 10-fold by day 8 (Fig. 2a, top). As well as proliferation, \(\alpha\)GC also induced increasing activation of iNKT cells over time (Fig. 2a, bottom). The lack of relative expansion of \(\alpha\)GC-treated iNKT cells on day 4 might be more apparent rather than real because upon \(\alpha\)GC exposure, the TCR of iNKT cells is known to be initially down-regulated but re-expressed later (21, 22). In the presence of vehicle, the relative frequency of iNKT cells during the 8-day MLR did not change significantly from baseline (Fig. 2a, top). Despite the lack of significant proliferation, vehicle-treated iNKT cells were progressively activated between days 0, 4, and 8 of the MLR (Fig. 2a, bottom). iNKT cells therefore are activated during the course of the MLR in the absence of \(\alpha\)GC, presumably by natural ligands presented to them by CD1d-expressing APC, although they do not

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**FIGURE 3.** iNKT cell depletion attenuates in vitro alloresponse. *a*, iNKT cell depletion of CD3\(^{+}\) T cells by flow sorting. Negatively selected CD3\(^{+}\) cells with a purity of \(\approx 90\%\) (data not shown) were stained with anti-TCR V\(\alpha\)24 and V\(\beta\)11 mAbs. Using a FACSDiva flow sorter, TCR V\(\alpha\)24\(^{-}\)V\(\beta\)11\(^{+}\) NKT cells were depleted from CD3\(^{+}\) cells. Pre- and postsorting analysis of at least \(5 \times 10^6\) events revealed that the procedure consistently generated highly iNKT cell-depleted CD3\(^{+}\) cells and highly purified iNKT cells. In addition, CD3\(^{+}\) iNKT cell-replete cells were sorted on the basis of their physical characteristics (data not shown). *b*, iNKT cell-replete MLR (indicated as CD3\(^{+}\)) using T cells from responder 49 against a panel of five stimulators was compared with iNKT cell-depleted MLR (indicated as CD3\(^{+}\)NKT\(^{-}\)) (left). In total, MLR of four responders were tested against three to five stimulators (each panel repeated twice with similar results), and in every responder to stimulator pair, iNKT cell depletion significantly diminished the alloreactive response ranging from 36 to 85\% (\(p < 0.001\); right). Data are presented as mean \(\pm\) SEM of triplicate assays. *c*, In accordance with the proliferation data, IFN-\(\gamma\) secretion was comparably reduced in iNKT cell-depleted MLR compared with baseline. No IL-4 was detected (representative of seven independent MLR).
proliferate significantly. On day 8 of the same series of MLR, we studied IFN-γ production by iNKT cells as well as total T cells. IFN-γ production was higher in iNKT cells treated with αGC rather than vehicle and, importantly, IFN-γ production by total T cells was considerably higher in the presence of αGC (Fig. 2b). In addition, in the presence of αGC there was increased activation of non-iNKT cells as determined by forward scatter characteristics (Fig. 2b) and CD69 expression (data not shown). These findings provide further evidence that increased reactivity of MLR in the presence of αGC does not merely reflect activation of iNKT cells but is primarily due to activation of alloreactive T cells.

**Role of iNKT cells in the absence of exogenous ligand**

The findings discussed above suggest that human iNKT cells are an integral part of the in vitro alloresponse and are required for its efficient development. This predicts that depletion of iNKT cells would attenuate the alloreactivity. To test this, we performed 4-day MLR using either iNKT cell-replete or cell-depleted responders. Rigorous iNKT cell depletion of negatively selected T cells was performed by staining with anti-TCR Vα24 and Vβ11 mAbs followed by flow sorting (Fig. 3a). iNKT cell-replete T cells were also flow sorted on the basis of their physical characteristics. To rule out preactivation of iNKT cells triggered by staining with the anti-TCR Vα24 and Vβ11 mAbs we established that engagement of the invariant TCR by the anti-TCR mAbs during the sorting procedure did not have a mitogenic effect on iNKT cells and did not alter their Th1/Th2 cytokine secretion balance (data not shown). When iNKT cells were depleted, the MLR reactivity of responder 49 against a panel of stimulators decreased by ~65% (Fig. 3b, left). In an extended panel in which we tested another three responders against three to five stimulators, the proliferation rate in iNKT cell-depleted compared with iNKT cell-replete MLR was reduced by (58.8 ± 24%; p < 0.001) in every responder to stimulator pair (Fig. 3b, right). This suppressive effect was mirrored by a comparable reduction of IFN-γ secretion in the supernatants of the iNKT cell-depleted MLR compared with baseline (by 43.2 ± 15.2%; n = 7; p < 0.001) (Fig. 3c). No IL-4 was detected. The effect of iNKT cell depletion was observed in MLR performed with varying R:S ratios (Fig. 4a) and also in longitudinal MLR (Fig. 4b). Finally, add-back of highly purified fresh iNKT cells to iNKT cell-depleted MLR restored proliferation close to the levels of the baseline iNKT cell-replete MLR (Fig. 4c). These findings suggest that although iNKT cells are not essential, they do contribute to the development of full MLR reactivity.

**Role of CD1d in MLR**

CD1d is the restricting element of iNKT cells and is expressed on monocytes, B cells, and APC. We therefore tested the effect of anti-CD1d mAb on the MLR. Anti-CD1d caused a dose-dependent inhibition of MLR as compared with Ig isotypic control (Fig. 5a) and anti-CD1d used at a fixed dose inhibited MLR at different R:S ratios (Fig. 5b). In longitudinal MLR, T cell proliferation and activation as determined by CFSE and HLA-DR staining, respectively, were significantly and dynamically decreased in the presence of anti-CD1d compared with Ig isotypic control (Fig. 5c). Further, T cells stimulated in primary MLR in the presence of anti-CD1d were less responsive in secondary MLR as compared with T cells derived from primary MLR treated with isotypic Ig control (Fig. 5d), suggesting that CD1d blocking may have rendered responder T cells anergic.

**CD1d is required for the iNKT cell-mediated effect on MLR**

In principle, the effect of anti-CD1d mAb on MLR might reflect either inhibition of an enhancing effect of NKT cells on the MLR or enhancement of an inhibitory effect. To discriminate between these two effects, MLR were performed using iNKT cell-replete or cell-depleted responders in the presence of anti-CD1d, anti-HLA class II, or Ig isotype (Fig. 6). As expected, the iNKT cell-replete MLR was inhibited by anti-CD1d and by anti-HLA class II mAbs either alone or in combination. iNKT cell depletion resulted in reduction of proliferation but the presence of anti-CD1d in the iNKT cell-depleted MLR did not result in any further significant reduction of proliferation. These results indicate that CD1d is required for the iNKT cell-mediated effect on MLR.
Detective effect of irradiation are protected from acute GVHD (10–12). This pro-

cell proliferation in the secondary as well as the primary 4-day MLR. Comparison of the primary with the 2-day MLR against the same stimulators at a R:S ratio of 1:2 equal numbers of T cells were placed in a secondary MLR against the same stimulators at a R:S ratio of 1:2 but without mAb. Comparison of the primary with the secondary MLR revealed that CD1d treatment reduces T cell proliferation in the secondary as well as the primary MLR. Each result is representative of three independent MLR.

FIGURE 5. Role of CD1d in the MLR. a, MLR were set up at a R:S ratio of 1:2 in the presence of varying concentrations of anti-CD1d or IgG. At an anti-CD1d concentration of as low as 5 μg/ml, MLR reactivity was significantly reduced. b, MLR performed in the presence of anti-CD1d 40 μg/ml was significantly inhibited at varying R:S ratios as compared with MLR treated with medium only. c, MLR were performed in the presence of anti-CD1d or IgG (40 μg/ml each). Proliferation and activation as assessed by CFSE and HLA-DR staining, respectively, were monitored at different time points by flow cytometry. Significant reductions in both proliferation and activation of T cells were observed over time in the presence of anti-CD1d as compared with anti-IgG. d, Proliferation was measured in primary 4-day MLR set up at a R:S ratio of 1:2 and treated with either anti-CD1d or IgG isotype (20 μg/ml each). After 2 days of rest, equal numbers of T cells were placed in a secondary MLR against the same stimulators at a R:S ratio of 1:2 but without mAb. Comparison of the primary with the secondary MLR revealed that CD1d treatment reduces T cell proliferation in the secondary as well as the primary MLR. Each result is representative of three independent MLR.

Discussion

Invariant NKT cells are emerging as an important subset of immunoregulatory cells. Unlike the exclusively suppressive immunomodulation mediated by the CD4+CD25+ subset of T cells (23), they either enhance or inhibit an Ag-specific immune response through direct interaction with APC (1). This is the first report dissecting the role of human iNKT cells (as defined by the coexpression of the TCR Vα24 and Vβ11 chains), CD1d (the re-

stricting element of iNKT cells), and αGC (the iNKT cell ligand) in the alloresponse. Our results suggest that the iNKT cell/CD1d axis positively regulates the alloresponse in humans. This idea is consistent with an earlier report documenting the ability of human iNKT cell clones to drive maturation of DC that can subsequently support efficient alloreactive responses (24). It is also consistent with studies showing that like the alloresponse, other Th1 responses against a variety of pathogens (for example, CTL responses against viruses) can diminish in the absence of iNKT cells or CD1d (25, 26). Conversely, in the presence of αGC, Th1 innate and adaptive immune responses are considerably enhanced in an iNKT cell- and CD1d-dependent manner (6, 7).

Our data contrast with a number of in vivo studies in which iNKT cells appear to inhibit rather than enhance the alloresponse. In murine models of acute GVHD involving myeloablative irradiation of the host, animals that receive αGC injections at the time of irradiation are protected from acute GVHD (10–12). This protective effect of αGC requires the presence of host iNKT cells and CD1d and is mediated by IL-4 secreted by host cells (10, 11). In our studies, in an attempt to mimic this in vivo set up, the stimu-

lators (PBMC) of the MLR were irradiated and pulsed with αGC. However, we consistently observed increased reactivity of the MLR both in terms of proliferation and IFN-γ secretion, an effect that was iNKT cell-dependent. Crucially, no IL-4 was detected at any time point tested during a 4-day MLR. This is in line with the observation that administration of αGC-loaded DC to normal hu-

man subjects is associated with increased IFN-γ and IL-12 but decreased IL-4 levels in the serum (27).

A second group of studies have reported that protection from lethal acute GVHD in the context of a nonmyeloablative, toler-

ance-inducing regimen is associated with persistence of CD1d/ αGC tetramer-positive host iNKT cells (13, 14). Similarly, donor-

derived NKT cells (identified as NK1.1+ T cells in C57BL/6 and DX5+ cells in BALB/c mice) protect from lethal acute GVHD after their adoptive transfer to a lethally irradiated host (16, 28). IL-4 and IL-10 seem to be the cytokine mediators of these effects (16, 28). However, NK1.1+ cells were not implicated as being the regulatory T cells that protect from acute GVHD following donor lymphocyte infusion (29, 30).

By performing MLR in the presence and absence of human peripheral blood iNKT cells, we established the importance of re-

sponder iNKT cells in enhancing the in vitro alloresponse in steady state and without exogenous pharmacological activation. This ef-

duct was documented by the specific decrease in proliferation and IFN-γ production in iNKT cell-depleted MLR and by restoration of these parameters upon adding back highly purified fresh iNKT cells. Furthermore, the effect of iNKT cells on the alloresponse was clearly CD1d-dependent. Interestingly, the degree of inhibition of the MLR upon iNKT cell depletion was remarkably uniform for a given responder tested against a panel of stimulators but
in vivo alloresponse. It is possible that because only 50–70% of human in vitro alloresponse and the opposite effect in the murine (31–33). Initial activation of iNKT cells requires a CD1d-dependent response have been studied in in vivo models of microbial infections expressed NK1.1 but are not stained with the CD1d/H9251/1/H11001/24 genes. The structure of the natural ligands driving activation of iNKT cells can be activated by both exogenous and natural ligands structurally resembling αβGC (11), whereas human in vivo studies (24 and this work) would favor iNKT cell depletion of donor T cells. Studies of the dynamics of iNKT cell activation in the setting of human acute GVHD will be beneficial in understanding more on the role of iNKT cells in vivo and how to exploit them for therapy.

In conclusion, we have shown that iNKT cells are naturally activated during the course of in vitro human alloreactivity in a CD1d-dependent manner and although not essential they contribute to the mounting of an efficient alloresponse.

Disclosures
The authors have no financial conflict of interest.

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