

# **Graft invariant natural killer T cell dose predicts risk of acute graft-versus-host disease in allogeneic hematopoietic stem cell transplantation**

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## **Abstract**

Invariant NKT (iNKT) cells are powerful immunomodulatory cells that in mice regulate a variety of immune responses including acute graft-versus-host disease (aGVHD). However, their clinical relevance and in particular their role in clinical aGVHD are not known. We studied whether peripheral blood stem cell (PBSC) graft iNKT cell dose impacts on the occurrence of clinically significant grade II-IV aGVHD in patients (n=57) undergoing sibling, HLA-identical allogeneic hematopoietic stem cell transplantation (HSCT). In multivariate analysis, CD4- iNKT cell dose was the only graft parameter to predict clinically significant aGVHD. The cumulative incidence of grade II-IV aGVHD in patients receiving CD4- iNKT cell doses above and below the median were 24.2% and 71.4% respectively (p=0.0008); low CD4- iNKT cell dose was associated with a relative risk for grade II-IV aGVHD of 4.27 (p=0.0023; 95% CI:1.68-10.85). Similarly, patients with grade 0-I aGVHD received significantly higher CD4- iNKT cell doses (p=0.001). Consistent with a role of iNKT cells in regulating aGVHD, in mixed lymphocyte reaction assays, CD4- iNKT cells effectively suppressed T cell proliferation and interferon- $\gamma$  secretion. In conclusion, higher doses of CD4- iNKT cells in PBSC grafts are associated with protection from aGVHD. This effect could be harnessed for prevention of aGVHD.

## **Introduction**

Invariant NKT cells are a subset of rare but powerful immunomodulatory T cells that are highly conserved between humans and mice<sup>1</sup>. They are selectively activated by glycolipids such as the prototypic ligand alpha-galactosylceramide ( $\alpha$ GC) presented by CD1d and are characterised by an invariant TCR $\alpha$  pairing with a diverse TCR $\beta$  chain (TCRV $\alpha$ 24J $\alpha$ 18 and TCRV $\beta$ 11 in humans)<sup>1</sup>. iNKT cells comprise two main subsets, CD4+ and CD4- cells, which in humans have distinct cytokine secretion profiles<sup>2</sup>. While the ability of murine iNKT cells to modulate immune responses against pathogens, in autoimmunity and in alloreactivity including experimental acute graft-versus-host disease (aGVHD) is firmly established<sup>3</sup>, the functional role, if any, of human iNKT cells in physiology and disease is ill-defined<sup>4</sup>.

Acute GVHD is the main source of treatment-related morbidity and mortality in patients receiving a T cell-replete allogeneic HSCT. It is caused by alloreactive donor T cells that are activated by host antigen presenting cells (APC) as a result of minor or major histocompatibility antigen disparity between donor and recipient<sup>5;6</sup> and subsequently target recipient tissues such as the skin, liver and gut<sup>6</sup>. The critical role of T cells as effectors of aGVHD is highlighted by the dramatic reduction in the incidence and severity of aGVHD in patients receiving T cell-depleted or syngeneic allografts<sup>7</sup>. Since T cell depletion of the graft is also associated with an increased risk of leukaemia relapse and infections, research has also focused on identifying other cellular components of the graft that impact on the incidence and severity of aGVHD. Indeed, the impact of the graft content of several immune effectors such as T, NK<sup>8</sup> and more recently B cells<sup>9</sup> as well as of the CD4+CD25hiFoxP3+ T regulatory cells (Tregs)<sup>10;11</sup> on the risk of aGVHD has been studied extensively.

The role of graft iNKT cells on the risk of aGVHD has not been investigated in humans. By contrast, in murine models, both recipient and donor iNKT cells were shown to effectively protect against experimental aGVHD. In a model involving lymphoablation with total lymphoid irradiation (TLI) and anti-thymocyte globulin (ATG), recipient iNKT cells preferentially survive due to radioresistance, secrete IL-4 and thus inhibit aGVHD<sup>12</sup>. In line

with these findings, ATG/TLI conditioning was shown to be associated with reduced incidence of aGVHD in humans<sup>13</sup>. Remarkably, iNKT cells in G-CSF-mobilised grafts were shown to protect from experimental aGVHD while enhancing the graft-versus-leukaemia (GVL) effect<sup>14</sup> and in vitro expanded donor iNKT cells alleviate aGVHD in a major histocompatibility complex (MHC) haploidentical setting<sup>15;16</sup>. Recent work also demonstrated the ability of unmanipulated, adoptively transferred donor iNKT cells to protect from experimental aGVHD without prior in vitro expansion<sup>17</sup>.

To directly test whether the protective role of donor iNKT cells shown in pre-clinical models also holds true in clinical allogeneic HSCT, we studied the impact of the dose of graft iNKT cells on the incidence and severity of aGVHD after a T-cell replete allogeneic HSCT from HLA-identical sibling donors.

## **Patients and Methods**

**Donors.** The research protocol was approved by the Imperial College Healthcare NHS Trust research ethics committee and all participants gave written informed consent. We analysed the frequency of effector and regulatory lymphocytes in cryopreserved samples of 78 sibling donor, G-CSF-mobilised peripheral blood stem cell (PBSC) grafts used for allogeneic HSCT between 1998 and 2011. In all cases, stem cell mobilisation, collection and storage of the graft were performed according to the same, JACIE-approved standard operating procedures. aGVHD diagnosis and grading criteria were as described<sup>18</sup>.

**Flow-cytometry and flow-sorting.** Staining of PBSC graft cells was performed at 4°C for 20min in the presence of directly fluorescently-conjugated monoclonal antibodies (mAbs). Cells were resuspended in phosphate buffer saline (PBS) plus 0.5% bovine serum albumin. Fc blocking reagent (Miltenyi Biotec, Germany) was used to reduce non-specific staining and 4',6-diamidino-2-phenylindole (DAPI) was used for dead cell exclusion. The mAbs used were anti-TCRV $\alpha$ 24-PE, TCRV $\beta$ 11-FITC (Beckman Coulter, High Wycombe, UK), CD4-PE-Cy7, CD161-Allophycocyanin, CD34-PerCP, CD16-FITC, CD8-Allophycocyanin (BD Biosciences, Oxford, UK), CD3-eFluor450, CD56-PE, CD19-Allophycocyanin-eFlour780 (eBioscience, San Diego CA). For intracellular FoxP3 staining, after surface staining, cells were fixed, permeabilized and stained using buffers and an anti-human FoxP3-FITC mAb (clone PCH101) from eBioscience (San Diego, CA), according to manufacturer's instructions. In parallel, samples were stained with appropriate isotypic controls. Samples were analysed in FACSCalibur and LSR Fortessa analysers, whereas FACS sorting was performed using a FACSDiva sorter (BD Biosciences). Data analysis was performed with the FlowJo software (Tree Star Inc. Oregon, USA). Because iNKT cells is a rare cell population, either a minimum of  $0.5 \times 10^6$  CD3+ cells or 200 iNKT cells were recorded to facilitate accurate calculation of the total, CD4- and CD4+ iNKT cell frequencies.

**Graft cellular component frequency and dose.** The frequencies of CD34+ cells, total CD3+ T cells, CD4+ and CD8+ T cells, CD19+ B cells and CD3-CD56+CD16+/- NK cells were determined by flow cytometry as a percentage of the total live cells. CD3+CD4+CD25<sup>hi</sup>FoxP3+ Tregs, CD3+CD161+ T cells, total, CD4+ and CD4-TCRV $\alpha$ 24+TCRV $\beta$ 11+ iNKT cell frequencies are presented as percentage of the total T cells (**Suppl Figure 1**). Infused cell doses of cellular components were calculated on the basis of the CD34+ cell dose infused to the patient.

**CD3+ T cell selection.** Buffy coats from normal blood donors were obtained from the North London Blood Transfusion Service and peripheral blood mononuclear cells (PBMC) were isolated after layering over Ficoll (Axis Shield Diagnostics, Cambridgeshire, UK). For negative selection of CD3+ cells the StemSep™ Human T Cell Enrichment Cocktail (StemCell Technologies, Meylan, France) was used as per manufacturer's instructions. Purity of CD3+ cells was always >95% (not shown).

**iNKT cell expansion.** Peripheral blood mononuclear cells (PBMC) were placed in 24 well plates at  $1-2 \times 10^6$  cells/ml in complete T cell medium (TCM) consisting of RPMI-1640 without L-glutamine (Invitrogen, Paisley, UK) supplemented with 5% heat inactivated human serum obtained from the North London Blood Transfusion Service, penicillin + streptomycin (Sigma, Dorset, UK) and L-glutamine (Sigma, Dorset, UK).  $\alpha$ GC was added at 100ng/ml; IL-2 was added on day 3 at 10u/ml and every 3-4 days thereafter. Cells were harvested on day 10 for further experiments without testing for cytokine or perforin secretion.

**Mixed lymphocyte reactions (MLR) and proliferation assays.** For MLR,  $3-5 \times 10^4$  negatively selected CD3+ cells (responders) were placed in triplicates against equal numbers of autologous or allogeneic (stimulators) irradiated (3000rad) PBMC in 96-well plates for 96hrs.  $^3\text{H}$ -thymidine (Amersham Biosciences, Buck, UK) was added at 1 $\mu$ Cu/well for the last 16hrs of the MLR. Proliferation was measured using a liquid scintillation counter (Wallac beta counter).

**Cytokine ELISA assays.** The Quantikine kit (all from R&D Systems, Oxon, UK) was used as per the manufacturer's instructions for the measurement of interferon- $\gamma$  in the supernatants of the MLRs.

**Transwell experiments.** To determine whether the suppressive effect was contact-dependent, MLR were set up in a transwell system (transwells of pore size 0.4 $\mu$ m, Costar, High Wycombe, UK) such that 3x10<sup>4</sup>/well FACS-sorted responder CD4<sup>+</sup> or CD4<sup>-</sup> iNKT cells were either placed in the lower chamber along with 3x10<sup>5</sup>/well responder CD3<sup>+</sup> cells and 6x10<sup>5</sup>/well irradiated stimulator cells or they were segregated from the responder cells by being placed in the top chamber. An MLR where responders and stimulators were mixed in the lower chambers without NKT served as the baseline. After 96 hrs equal volumes of cells were removed and set up in triplicate in 96-well plates for a primary proliferation assay. <sup>3</sup>H-thymidine was added at 1 $\mu$ Ci/well for the final 16 hours when proliferation was measured in a liquid scintillation counter.

**Dendritic cell generation.** For myeloid dendritic cell generation, CD14-selected cells were initially plated at 1x10<sup>6</sup>/ml in a 24 well plate in medium consisting of RPMI-1640 (Invitrogen, Paisley, UK), 10% heat inactivated human serum (normal screened donors, North London Blood Centre) supplemented with 1% penicillin/streptomycin (Sigma, Dorset, UK) and L-glutamine (Sigma, Dorset, UK). GM-CSF (Sigma, Dorset, UK) at 50ng/ml and IL-4 (Sigma, Dorset, UK) at 1000U/ml were added to each well to generate immature DC. After 4 days in culture DC were matured with TNF $\alpha$  (Sigma, Dorset, UK) at 50ng/ml and cultured for a further 40 hours. Maturity was checked by flow cytometry using the following antibodies: CD14-FITC, CD80-PE, CD83-PE, CD86-PE and HLA-DR-Allophycocyanin (BD Biosciences, Oxford, UK). Mature DCs were identified by the increased CD80, CD83, CD86 and HLA-DR and decreased CD14 expression (not shown).

**Cr<sup>51</sup> release assay.** Target cells were resuspended in TCM, labelled with Cr<sup>51</sup> (100  $\mu$ Ci/10<sup>7</sup> cells) for 1hr at 37°C, 5% CO<sub>2</sub> and after three washes with PBS they were seeded into V-

shaped 96-well plates at a concentration of  $10^4$  cells/well. Effector CD4- iNKT cells were added at different effector to target cell (E:T) ratios in triplicates. For measurement of the spontaneous release of  $\text{Cr}^{51}$ , TCM was added to target cells instead of iNKT cells and for the measurement of total release, Triton-X (1% final concentration) was added to target cells instead of iNKT cells. After 4hrs of incubation at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ,  $\text{Cr}^{51}$  release was measured using a beta counter. The  $\text{Cr}^{51}$  specific release was calculated using the following formula: % specific  $\text{Cr}^{51}$  release = (release of test - spontaneous release) / (total release - spontaneous release)

**Statistical analysis.** Cellular subset variables were divided into low and high dose groups using the median value. Probabilities of grades 0-I and II-IV aGVHD were calculated using the cumulative incidence procedure, with relapse and death without aGVHD within 180 days (n=4) treated as competing events. Univariate comparisons for both cellular subset and clinical variables were made using Gray's test<sup>19</sup>. Variables significant at the  $p < 0.1$  level, were entered into a regression model by Fine and Gray<sup>20</sup>, and a backward stepping procedure was utilised to find the best model. Graft cell subset doses infused were compared between the aGVHD groups using the Mann-Whitney test. Statistical analyses were carried out using the software packages SPSS v19 and R. P values  $< 0.05$  were deemed statistically significant.



## **Results**

### **Frequency distribution of effector and regulatory lymphocytes in G-CSF-mobilised PBSC donor grafts**

The frequencies of total, CD4+ and CD4- iNKT cells, CD4+, CD8+ and total T cells, B cells, NK cells as well as Tregs were analysed by multicolour flow-cytometry in 78 normal donor G-CSF-mobilised PBSC grafts (**Suppl Figure 1**). In addition, the frequency of CD3+CD161+ regulatory T cells previously shown to be enriched in non-invariant, CD1d-restricted T cells that can suppress in vitro alloreactivity was determined<sup>21</sup>. The frequencies and the absolute doses of the graft cell populations infused to patients at the time of peripheral blood stem cell transplant (PBSCT) are shown in **Table 1**. A striking feature of iNKT cells that distinguishes them from the other cellular components is their distinct skewed distribution pattern and dramatic (up to a 1000-fold) variation of their frequency (**Suppl Figure 2 and Table 1**). This variation is independent of sex and age (**Suppl Figure 3**). Finally, there was no correlation between frequency of iNKT cells with that of Tregs or any of the other T cell subsets (data not shown).

### **Patient characteristics**

To analyse the impact of clinical variables and graft cellular components on aGVHD, out of the 78 studied donors we selected a cohort of 57 consecutive donor-recipient pairs in whom a G-CSF mobilised PBSC graft was used for a T cell-replete, sibling HLA-identical, either myeloablative (n=41) or reduced intensity conditioning (n=16) allogeneic HSCT for haematological malignancies. These included chronic myeloid leukaemia (CML, n=18), acute myeloid leukaemia (AML, n=23), acute lymphoblastic leukaemia (ALL, n=6), myelodysplastic syndrome (MDS, n=5), multiple myeloma (MM, n=2), myelofibrosis (n=2) and Sezary syndrome (n=1) (**Suppl Table 1**). None of the 57 patients received either ATG or alemtuzumab in the preparative regimen. aGvHD prophylaxis was cyclosporine-A plus methotrexate. The inclusion criteria aimed at minimising confounding factors that would

mask the effect of graft iNKT cells on the incidence and severity of aGVHD. Therefore, T cell-depleted HSCT were not included.

### **PBSC graft content and acute graft-versus-host disease**

Since iNKT cells uniquely amongst the lymphocyte subsets showed a distinct skewed distribution pattern we predicted that in conjunction with the marked variability of their frequency in PBSC grafts, iNKT cell graft dose could be an important determinant of the incidence and severity of aGVHD.

The cumulative incidence of grade II-IV aGVHD for the 57 patients receiving a G-CSF-mobilised PBSC, T cell-replete, HLA-identical, sibling donor allograft was 47.3% at 180 days post-transplant (**Fig 1A**). The impact of the dose of the principal cellular components of the PBSC graft and of clinical variables (**Table 2**) previously shown to impact on the incidence of grade II-IV aGVHD<sup>22-26</sup> were analysed.

In univariate analyses, total iNKT, CD4+ iNKT and CD4- iNKT cell doses and the diagnosis of CML were significantly ( $p < 0.05$ ) associated with grade II-IV aGVHD (**Figure 1B-D and Table 2**). The cumulative incidence of grade II-IV aGVHD at 180 days post HSCT for recipients receiving cell doses below and above the median for total iNKT cells was 64.3% vs 31% ( $p = 0.014$ ), for CD4+ iNKT cells 67.5% vs 27.6% ( $p = 0.002$ ), and for CD4- iNKT cells 71.4% vs 24.2% ( $p = 0.0008$ ). In line with these results, iNKT cell doses were the only graft variables that were statistically different between patients who subsequently developed grade II-IV and those with grade 0-I aGVHD (**Suppl Table 2**). Unlike iNKT cells, graft Treg dose did not predict grade II-IV aGVHD- a similar cumulative incidence of aGVHD was found in patients receiving cell doses below and above the median (50% vs 45%,  $p = 0.6$ ; **Table 2**).

Several clinical variables affecting the risk for aGVHD such as sibling versus unrelated donor, HLA disparities, stem cell source and prophylaxis regimen including the use of ATG or alemtuzumab were not relevant to our cohort of patients. Upon univariate analysis of other clinical variables previously shown to confer an increased risk of grade II-IV aGVHD in

sibling stem cell transplants, including recipient and donor age, use of TBI sex mismatched transplants and diagnosis of CML<sup>22-26</sup>, only the latter was found to impact on the occurrence of aGVHD (**Table 2**). Specifically, the cumulative incidence of grade II-IV aGVHD for patients with a diagnosis of CML was 61% compared with 41% for other malignancies (p=0.038).

Following multivariate regression analysis, CD4- iNKT cell dose and CML diagnosis were the only independent factors associated with the occurrence of grade II-IV aGVHD (**Table 3**). A low CD4- iNKT cell dose was associated with a relative risk (RR) of grade II-IV aGVHD of 4.27 (p=0.0023; 95% CI: 1.68-10.85), while diagnosis of CML conferred a RR of 2.54 (p=0.036; 95% CI: 1.06-6.04). Unlike aGVHD, we found no association between the iNKT cell dose infused and occurrence of moderate to severe chronic GVHD (data not shown).

### **Function of CD4- iNKT cells in the in vitro alloresponse**

To directly show that human iNKT cells are able to inhibit the alloresponse, as shown in murine studies in which  $\alpha$ GC-expanded donor iNKT cells ameliorate experimental aGVHD<sup>15,16</sup>, we tested the ability of  $\alpha$ GC-expanded human CD4+ and CD4- iNKT cell subsets to regulate the MLR, an in vitro correlate of the alloresponse. For this purpose, highly purified (**Suppl Figure 4**) normal donor CD4+ and CD4- NKT cells, autologous to responder T cells, were added to the allogeneic, HLA-mismatched MLR at an iNKT:responder (R) ratio of 10:1. While the effect of CD4+ iNKT cells was variable (**Suppl Figure 5**), addition of CD4- iNKT cells had a consistently inhibitory effect (p=0.0048; one sample z test) and titration of the CD4- iNKT cell numbers resulted in inhibition of the MLR in a dose-dependent manner (**Figure 2A-C**). On subsequent studies that focused on the function of CD4- iNKT cells, their addition reduced interferon- $\gamma$  secretion during MLR (**Figure 2D and Suppl Fig 6**) and in a transwell co-culture set up, their suppressive effect was found to be contact-dependent (**Figure 2E and Suppl Fig 6**). In line with this and their known selective expression of perforin<sup>2</sup>, CD4- iNKT cells efficiently lysed allogeneic mature

myeloid dendritic cells (DC), an effect abolished by the  $\text{Ca}^{++}$  chelator EGTA and consistent with cytotoxicity due to perforin (**Figure 2F and Suppl Fig 6**).

## **Discussion**

This study addresses an unresolved question in the biology of iNKT cells, namely whether they have any functional significance in humans<sup>4</sup>. Defining the role of iNKT cells in disease has been hampered by the difficulty of tracking them in time and in space during the course of human pathology such as autoimmunity and malignancy. Study of iNKT cells in donor PBSC grafts provides a unique opportunity to accurately identify and enumerate iNKT cells as well as other effector and regulatory immune cells in humans and thus define their role in aGVHD, a major source of morbidity and mortality in allogeneic HSCT. We addressed this important question by studying the impact of iNKT cells contained in G-CSF-mobilised PBSC grafts based on evidence from the murine model showing that a) G-CSF-mobilised iNKT cells protect from aGVHD and enhance the graft versus leukemia (GVL) effect<sup>14</sup> and b) adoptively transferred, unmanipulated<sup>17</sup> or in vitro  $\alpha$ GC-expanded<sup>15;16</sup> iNKT cells can ameliorate aGVHD.

By studying a larger cohort than in previous reports<sup>27</sup> we confirmed that graft iNKT cells are as rare and as variable in their frequency as peripheral blood iNKT cells and found that in terms of frequency, they are the most variable amongst all other graft cellular components. Consequently, for patients who would otherwise receive comparable doses of other cell types, there is a higher probability that they would receive considerably different doses of iNKT cells. Given the efficacy of donor iNKT cells in controlling murine aGVHD, we anticipated that higher doses of iNKT cells might also be protective in the clinical setting. Indeed, univariate analysis demonstrated the unique effect of total iNKT cells and subsets in predicting aGVHD. Multivariate analysis showed that a low median dose of CD4- iNKT cells was the only variable associated with grade II-IV aGVHD with a relative risk of 4.27. This level of risk prediction is one of the highest reported either for clinical parameters or graft cellular components and suggests a powerful influence of iNKT cell dose on the incidence and severity of aGVHD.

In contrast to previous reports<sup>22</sup>, clinical variables such as recipient age and conditioning regimen were not significant determinants of aGVHD in our study. This is very likely due to the small effect that each of these parameters exerts on the risk of aGVHD (generally RR <2) which our study was underpowered to detect. In line with previous reports<sup>22</sup>, CML diagnosis was associated with higher risk of aGVHD (**Table 2**) than other haematological malignancies.

To determine whether graft cellular subsets other than T cells might predict for the occurrence of aGVHD, the frequencies and contents of other effector and regulatory lymphocyte subsets have been intensively studied. In general, the reported effects of NK<sup>8</sup> and B cell<sup>28</sup> doses are either relatively small or not confirmed in all studies and this was also the case in our study. Our results are also in agreement with several other reports showing that the dose of T cells, the undisputed and most powerful effectors of aGVHD, does not predict risk of aGVHD<sup>29</sup>. This is consistent with the 'all-or-none' notion that alloreactive T cells induce aGVHD only when their dose exceeds a threshold of  $5\text{-}10 \times 10^4$  cells/Kg<sup>30</sup>.

Whether and how graft FoxP3+ Tregs might protect from aGVHD has also been investigated extensively. Although a protective effect of high graft Treg content was suggested in some<sup>10;11</sup> but not all<sup>31</sup> reports, we found no evidence that the Treg dose affects the risk of aGVHD. Nevertheless, two phase I clinical trials designed to test the safety of Treg infusion for prevention of aGVHD have already been reported<sup>32;33</sup>.

CD4- iNKT cells formed the subset primarily associated with the protective effect against aGVHD. Human CD4- iNKT cells differ functionally from their CD4+ counterparts in terms of cytokine and cytotoxicity profiles: they secrete higher amounts of interferon- $\gamma$  than IL-4 having thus a Th1 bias and express preferentially perforin<sup>2</sup>. Although both subsets inhibited MLR proliferation in our vitro alloreactivity studies, in line with their stronger association with protection from aGVHD in vivo, this effect was more consistently observed with CD4- iNKT cells, which in a contact-dependent manner displayed direct cytotoxicity against CD1d-

expressing mature myeloid DC. Furthermore, we previously showed that iNKT cells, despite the non-polymorphic nature of CD1d, can display alloreactivity, an effect that requires CD1d and invariant TCR interaction but is instructed by activating KIR interacting with yet to be identified APC ligands<sup>34</sup>. In this regard, iNKT cells resemble NK cells which in the context of donor-recipient KIR mismatch are activated and exert a selective GVL effect<sup>35</sup>.

We therefore propose that one possible mechanism for the protective effect of iNKT cells might involve donor CD4- iNKT cells interacting with host APC, the cells that orchestrate aGVHD in secondary lymphoid organs<sup>36</sup>. This interaction would lead to activating KIR-dependent iNKT cell activation, followed by perforin-mediated cytotoxicity of APC.

In the clinical setting, enhancing the iNKT cell dose by adding-back either ex vivo selected or in vitro expanded iNKT cells to iNKT cell-poor grafts might constitute a novel strategy for prevention of moderate to severe aGVHD. To this end, clinical grade protocols for iNKT cell selection and expansion are under development<sup>37</sup>.

In conclusion, this study shows that the dose of iNKT, and in particular of CD4- iNKT cells delivered with the PBSC graft in the context of HLA-identical T cell-replete allogeneic HSCT, is a significant determinant of the incidence and severity of aGVHD and provides a basis for developing new strategies for its prevention.

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**Author contribution:** AC performed research, analysed data, wrote the paper; SP performed research, analysed data; RS analysed data, wrote the paper; SC retrieved and analysed data; FD, ED, DMD, DM, DM, JP, AR and KR analysed data, contributed to writing of the paper; JD contributed reagents; JG contributed to writing of the paper; IR, analysed data, contributed to the writing of paper, supervised research; JA analysed data, contributed to writing of the paper; AK, designed and supervised research, analysed data, wrote the paper

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**Table 1.** PBSC graft cell subset frequencies<sup>a</sup> and cell doses<sup>b</sup> from 78 normal donors

n=78	Frequencies of graft cell subsets (%)		Doses of graft cell subsets infused	
	Mean±SD <sup>c</sup>	Median (Min-Max)	Mean±SD	Median (Min-Max)
<b>CD34+</b>	2.1±1.7	1.6 (0.4 - 12.6)	5.9±2.8	5.7 (1.1 - 16.8)
<b>NK cells</b>	3.6±2.5	3.2 (0.5 - 18.4)	13.2±12.3	10.5 (0.46 - 80.8)
<b>B cells</b>	11.9±6.5	10.5 (0.9 - 34.8)	46.1±52.1	33.9 (5.1 – 162.3)
<b>T cells</b>	45.6±11.4	45.30 (21.8 - 75.4)	170.5±119	145 (16.8 - 684.1)
<b>CD4+ T cells</b>	30.4±8.6	29.8 (12.6 - 56.5)	115±86.9	89.9 (8.8 - 511)
<b>CD8+ T cells</b>	13.1±5.8	11.8 (4.1 - 29.2)	47.7±34.6	36.8 (5.4 - 154)
<b>Tregs</b>	3.8±1.6	3.5 (0.9 - 8.2)	6.68±6.5	4.8 (0.4 - 41.3)
<b>CD3+CD161+</b>	12.7±6.9	12.6 (1.6 - 32.6)	10.1±11.3	6.4 (0.6 - 62.1)
<b>iNKT cells</b>	0.139±0.23	0.043 (0.001 - 1.07)	0.2±0.312	0.052 (0.002 -1.4)
<b>iNKT CD4-</b>	0.091±0.175	0.019 (0.001 - 0.76)	0.113±0.2	0.031 (0.001 - 1.04)
<b>iNKT CD4+</b>	0.061±0.12	0.015 (0.001 - 0.68)	0.091±0.175	0.025 (0.001 - 1.09)

<sup>a</sup>CD34+, NK, B, T, CD4+ and CD8+ T cell frequencies are shown as percentage of the total live cells. Tregs, CD3+CD161+ and iNKT cell frequencies are shown as percentage of the T cells.

<sup>b</sup>Cell doses are shown in 10<sup>6</sup> cells per Kg of body weight

<sup>c</sup>SD: Standard Deviation

**Table 2.** Clinical variables and PBSC graft cell subset doses<sup>a</sup> tested in univariate analysis.

<b>Variables</b>		<b>Cumulative incidence of grade II-IV aGVHD (95% CI)</b>	<b>p</b>
<b>TBI</b>	TBI <sup>b</sup> /Cy (n=39) vs non-TBI (n=18)	51% (34-66%) vs 39% (16-61%)	<b>0.26</b>
<b>Recipient age</b>	<47.2 vs ≥47.2 years	46% (27-63%) vs 48% (29-65%)	<b>0.91</b>
<b>Donor age</b>	<47.5 vs ≥47.5 years	46% (27-64%) vs 48% (29-65%)	<b>0.85</b>
<b>Sex mismatch</b>	yes (n=19) vs no (n=38)	50% (33-65%) vs 42% (20-63%)	<b>0.46</b>
<b>Disease risk<sup>c</sup></b>	Standard (n=16) vs High (n=41)	44% (19-66%) vs 49% (36-63%)	<b>0.74</b>
<b>Conditioning</b>	Myeloablative (n=41) vs RIC <sup>d</sup> (n=16)	44% (19-67%) vs 49%(33-63%)	<b>0.53</b>
<b>CML vs other</b>	n=18 vs n=39	61% (34-80%) vs 41% (25-56%)	<b>0.038</b>
<b>CD34+</b>	<5.66 vs ≥5.66	54% (33-70%) vs 41% (23-59%)	<b>0.52</b>
<b>NK cells</b>	<11.7 vs ≥11.7	39% (21-57%) vs 55% (35-71%)	<b>0.28</b>
<b>B cells</b>	<33.5 vs ≥33.5	57% (36-73%) vs 38% (20-55%)	<b>0.12</b>
<b>T cells</b>	<154 vs ≥154	54% (32-70%) vs 41% (23-58%)	<b>0.24</b>
<b>CD4+ T cells</b>	<91.2 vs ≥91.2	46% (27-64%) vs 48% (29-65%)	<b>0.71</b>
<b>CD8+ T cells</b>	<40.3 vs ≥40.3	57% (36-73%) vs 38% (20-55%)	<b>0.09</b>
<b>Tregs</b>	<4.9 vs ≥4.9	50% (30-67%) vs 45% (26-61%)	<b>0.6</b>
<b>CD3+CD161+</b>	<7.07 vs ≥7.07	46% (27-64%) vs 48% (28-65%)	<b>0.62</b>
<b>iNKT cells</b>	<0.057 vs ≥0.057	64% (43-79%) vs 31% (15-48%)	<b>0.014</b>

<b>CD4+ iNKT</b>	<0.026 vs ≥0.026	68% (46-82%) vs 26% (13-45%)	<b>0.002</b>
<b>CD4- iNKT</b>	<0.031 vs ≥0.031	71% (50-85%) vs 24% (10-41%)	<b>0.0008</b>

<sup>a</sup> Cell doses in 10<sup>6</sup> cells per Kg of body weight. Median values are shown.

<sup>b</sup> TBI: Total body irradiation; Cyclo: Cyclophosphamide

<sup>c</sup> Standard risk: CML chronic phase 1, AML/ALL standard risk in 1<sup>st</sup> complete remission. High risk: all other

<sup>d</sup>RIC: Reduced intensity conditioning



**Table 3.** Significant variables from a multivariate analysis describing the probability of developing severe aGVHD.

Variable	N	RR	CI (95%)	<i>p</i>
<b>Diagnosis</b>	57			
non CML		1		
CML		2.54	1.06-6.04	0.036
<b>CD4- iNKT cell dose</b>	57			
>0.031x10 <sup>6</sup>		1		
<0.031x10 <sup>6</sup>		4.27	1.68-10.85	0.0023

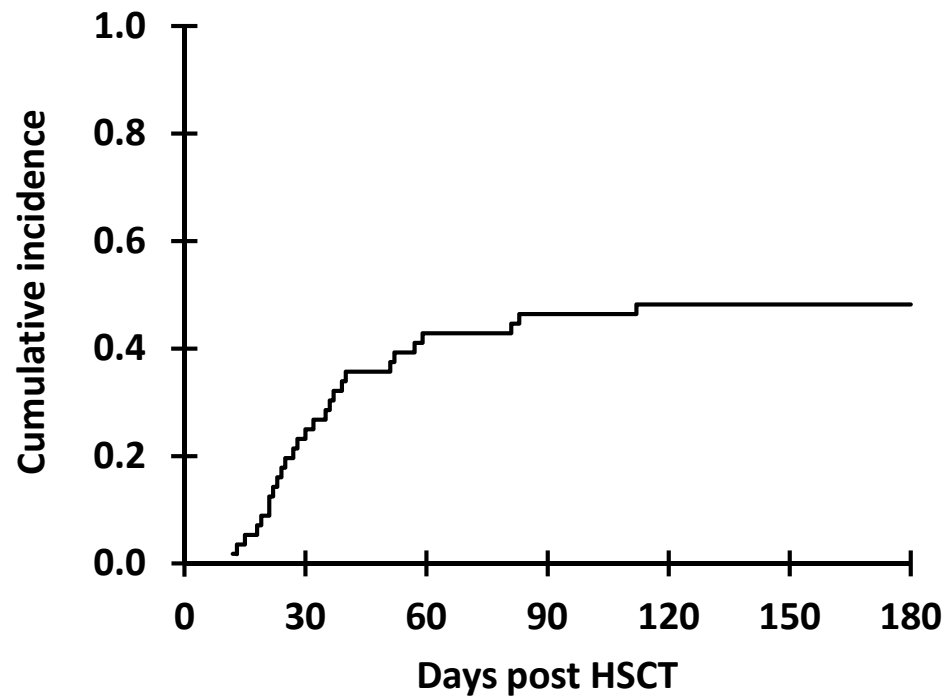
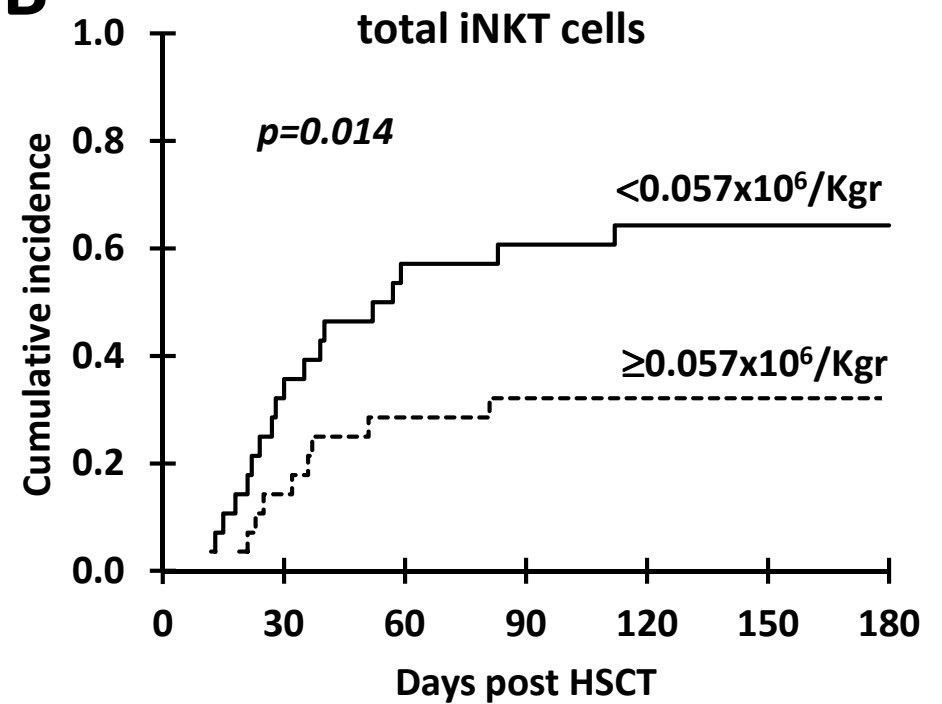
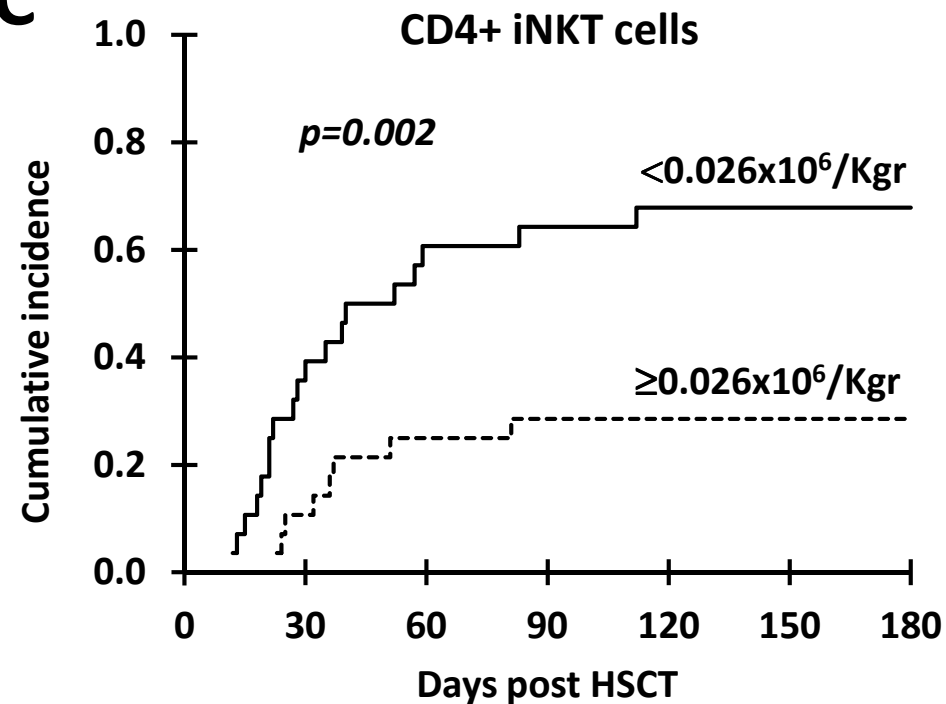
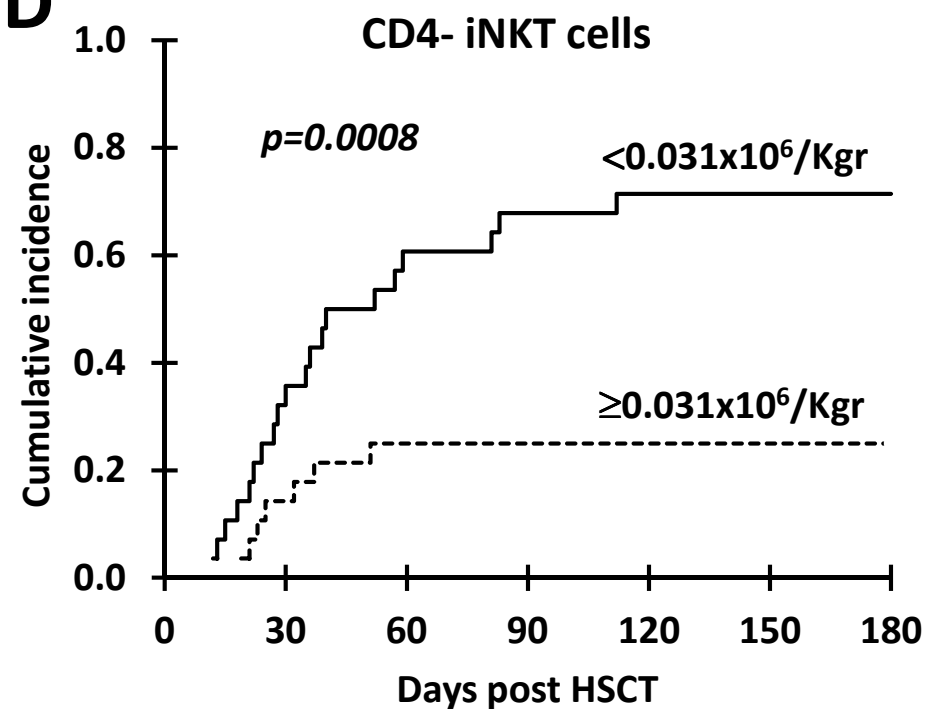
‘Best’ model from a forward stepping procedure that included the variables: CML diagnosis, CD8+ T cell dose, total iNKT cell dose, CD4+ iNKT cell dose and CD4- iNKT cell dose. Abbreviations RR = Relative Risk, 95%CI = 95% confidence interval.

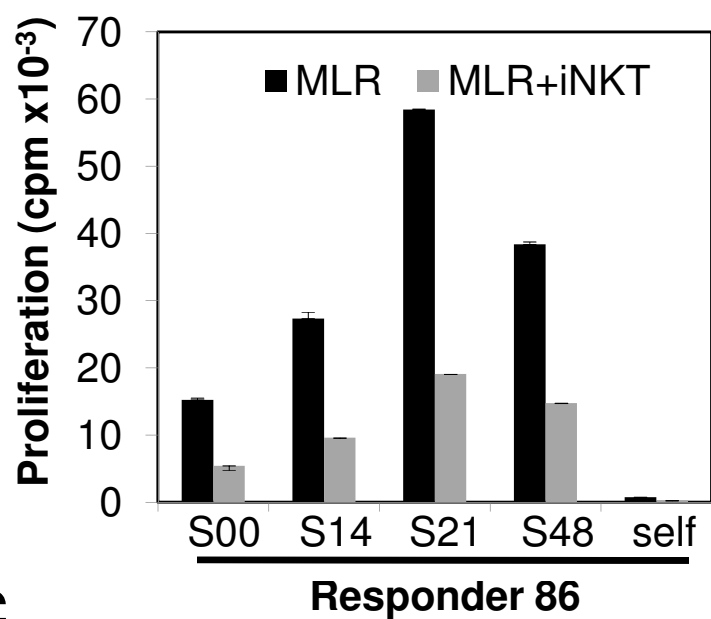
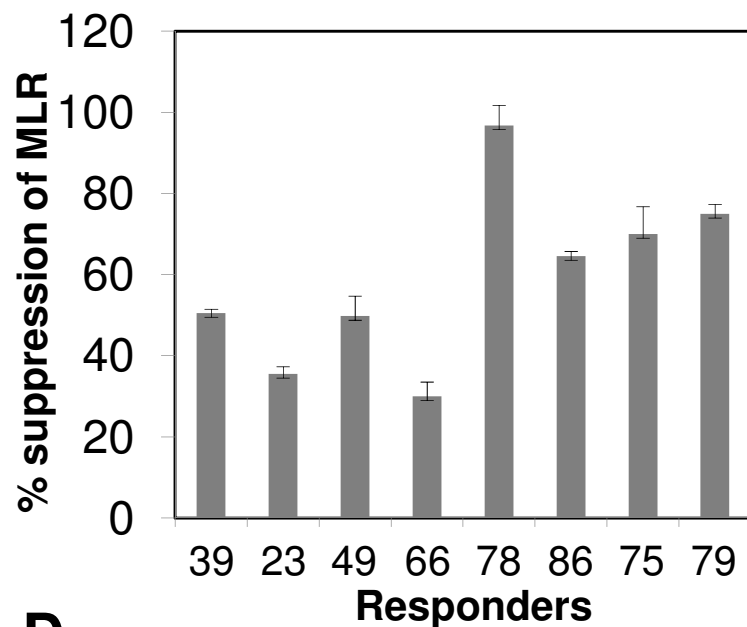
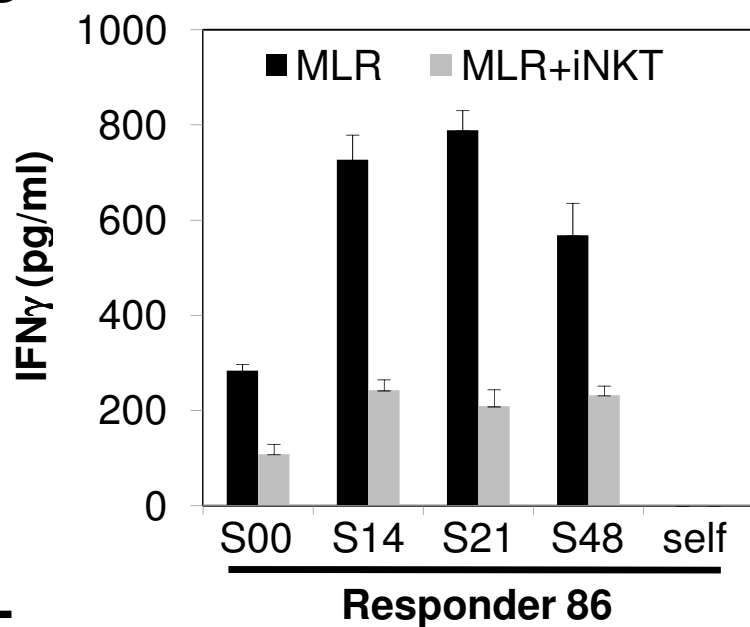
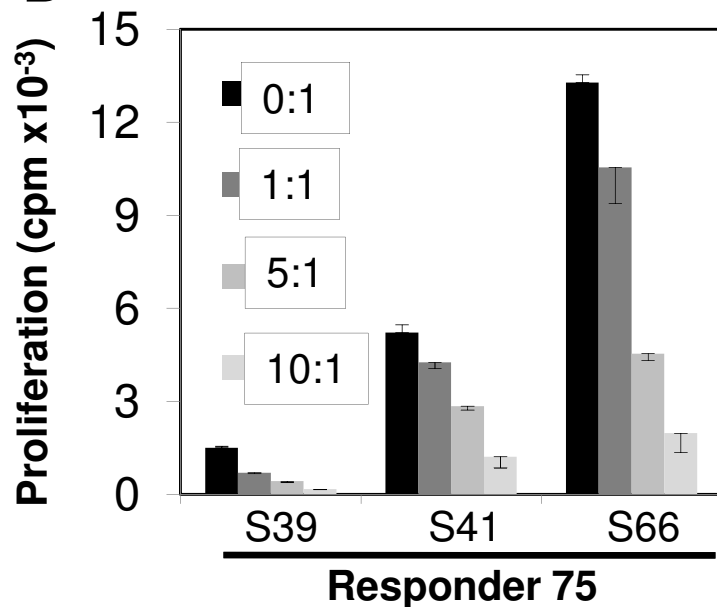
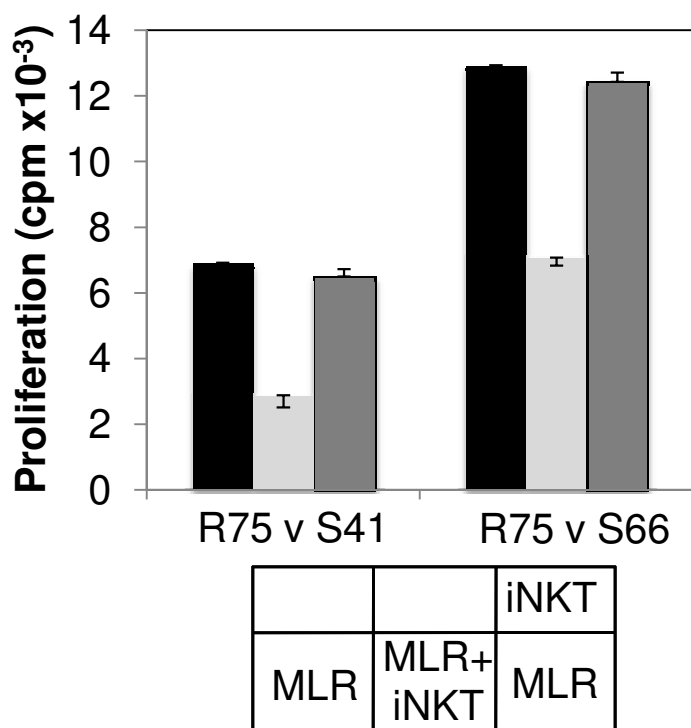
## **Figure Legends**

**Figure 1. Cumulative incidence of grade II-IV aGVHD.** Cumulative incidence of grade II-IV aGVHD in all patients **(A)** and in patients receiving below versus above the median dose of **(B)** total iNKT, **(C)** CD4+ iNKT and **(D)** CD4- iNKT cells.

**Figure 2. Effect of CD4- iNKT cells on MLR.** **A.**  $\alpha$ GC-expanded, highly purified CD4- iNKT cells from a normal donor (R86) were placed in MLR comprising autologous CD3+ T cells (responders; R) against allogeneic irradiated PBMC (stimulators; S) at iNKT: R:S ratio of 10: 1: 1 and compared with R:S (baseline) and autologous (self) MLR. Cell proliferation was measured by  $^3\text{H}$ -Thymidine incorporation 96hrs later. Bars represent means and error bars standard error of the mean (SEM) of triplicate assays. **B.** In a series of MLR involving 8 different responders, each placed against a panel of 3-4 allogeneic stimulators, addition of autologous CD4- iNKT cells resulted in a  $59\%\pm 8$  ( $p=0.0048$ ; one sample z test) decrease in proliferation compared to baseline MLR. **C.** Interferon- $\gamma$  ( $\text{IFN}\gamma$ ) secretion was significantly decreased upon addition of CD4- iNKT cells to the baseline MLR of R86 against the same panel of stimulators as in A. Data are shown as  $\text{mean}\pm\text{SEM}$  of triplicate assays. **D.** Dose-dependent inhibition of the MLR by CD4- iNKT cells. Responder 75 (R75) CD4- NKT cells were added in MLR at different iNKT : R ratios as shown. Data are shown as  $\text{mean}\pm\text{SEM}$  of triplicate assays. **E.** Contact-dependent inhibition of the MLR by CD4- iNKT cells. MLRs of R75 vs two stimulators were set up in a transwell co-culture system as shown at the bottom and described in material and methods. Contact of CD4- NKT cells with the responder and stimulator cells suppressed the MLR; when CD4- iNKT cells were separated from the MLR by the transwell membrane, cell proliferation was almost completely restored to the levels of the baseline MLR. Data are shown as  $\text{mean}\pm\text{SEM}$  of triplicate assays. **F.** CD4- iNKT cells are cytotoxic against allogeneic myeloid dendritic cells (DC). CD4- iNKT cells were cytotoxic against allogeneic DC in a  $\text{Cr}^{51}$  release assay at the iNKT : DC ratios of 1:1 and 10:1, an

effect that was abrogated by the  $\text{Ca}^{++}$  chelator EGTA. Data are shown as mean $\pm$ SEM of triplicate assays. See also Suppl Fig 6.

**A****B****C****D**

**A****B****C****D****E****F**