Human CD4+ invariant NKT lymphocytes regulate graft versus host disease

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ABSTRACT

Despite increasing evidence for a protective role of invariant (i) NKT cells in the control of graft-versus-host disease (GVHD), the mechanisms underpinning the regulation of the allogeneic immune response in humans are not known. In this study, we evaluated the distinct effects of human in vitro expanded and flow-sorted human CD4+ and CD4+ iNKT subsets on human T cell activation in a pre-clinical humanized NSG mouse model of xenogeneic GVHD. We demonstrate that human CD4+ but not CD4+ iNKT cells could control xenogeneic GVHD, allowing significantly prolonged overall survival and reduced pathological GVHD scores without impairing human T cell engraftment. Human CD4+ iNKT cells reduced the activation of human T cells and their Th1 and Th17 differentiation in vivo. CD4+ and CD4+ iNKT cells had distinct effects upon DC maturation and survival. Compared to their CD4+ counterparts, in co-culture experiments in vitro, human CD4+ iNKT cells had a higher ability to make contacts and degranulate in the presence of mouse bone marrow-derived DCs, inducing their apoptosis. In vivo we observed that infusion of PBMC and CD4+ iNKT cells was associated with decreased numbers of splenic mouse CD11c+ DCs. Similar differential effects of the iNKT cell subsets were observed on the maturation and in the induction of apoptosis of human monocyte-derived dendritic cells in vitro. These results highlight the increased immunosuppressive functions of CD4+ versus CD4+ iNKT cells in the context of alloreactivity, and provide a rationale for CD4+ iNKT selective expansion or transfer to prevent GVHD in clinical trials.

INTRODUCTION

Acute graft-versus-host disease (GVHD) occurs in up to 50% of patients who receive an allogeneic HLA-matched hematopoietic stem cell transplantation (allo-HSCT) and still represents a major cause of post-transplant morbidity and mortality.1 Thus, reducing aGVHD while preserving its beneficial anti-leukemic (GVL) effect remains the main challenge of allo-SCT. The pathophysiology of aGVHD, has been described as a multi-step process involving host antigen presenting cells (APC) as the main initiators and donor T cells as the major effectors of the allogeneic immune response.2 Acute GVHD has been mainly associated with a Th1 and Th17 T cell profile3 and can be down regulated by several subsets of immunoregulatory cells of potential therapeutic interest; among these, CD4+CD25highFoxP3regulatory T cells (Tregs) have been shown to prevent aGVHD following haplo-identical SCT.4 However, there are some concerns about the their potential impairment of the GVL effect.5,6 Invariant NKT cells (iNKT) represent another immunoregulatory T cell subset characterized by a CD1d-restricted invariant TCR that recognizes glycolipids presented by the non-classical MHC class Ib-like molecule CD1d.7 The hallmark of iNKT cells is a rapid production of a wide spectrum of cytokines and chemokines, as well as cytotoxic capabilities that enable them to regulate the immune response.8 Several groups have shown the ability of recipient or donor mouse iNKT cells to control GVHD, without affecting the GVL effect.9–14 In humans, our group and others previously reported that the graft content of iNKT cells and their post-transplant reconstitution are associated with a reduced risk of aGVHD whereas GVL effects were preserved.11–18

Although iNKT cells represent an evolutionary conserved CD1d-restricted subset of T cells, murine and human iNKT cells display major functional differences. There is also a very high discrepancy of proportions and differentiation stage of circulating iNKT cells between mice and humans. In peripheral human blood, iNKT represent a highly variable proportion of CD3+ T cells (0.001 to 1%) with a homogeneous late (stage 3) maturation state.19,20 In mice, they reach 0.2–1% of circulating CD3+ T cells, in which the three maturation stages...
are heterogeneously represented. Most importantly, while in mice, both CD4⁺ or CD4⁻ subsets can produce Th1 and Th2 cytokines, human iNKT cells are separated in two functionally distinct subsets: the CD4⁺ subset producing Th1 and Th2 cytokines (IL-4, IL-13, IFN-γ) and the CD4⁻ subset producing a more restricted Th1 (IFN-γ) profile. In mice, the protective effect of iNKT cells upon GVHD has been related to IL-4, and the induced expansion of Tregs. In humans, correlations have been described between the occurrence of acute GVHD and the graft content or the expansion capacities of iNKT cells, but their direct implication in the control of GVHD and their mechanisms of action had not been yet established. In addition, while in mice the CD4⁺ iNKT cell subset has been shown to control GVHD, the correlations made in humans between graft iNKT cells and the occurrence of aGVHD suggest a role for the CD4⁺ iNKT cell subset. Because CD4⁺ iNKT cells represent the predominant iNKT subtype in human grafts and in the recipient's peripheral blood after transplantation, their potential interest compared to their CD4⁺ counterparts in the control of alloreactivity needed to be explored.

In this study, we therefore compared the potential role and mechanisms of action of human CD4⁺ versus CD4⁻ iNKT subsets in the NSG mouse model of xenogeneic GVHD.

Results

Human CD4⁻ but not CD4⁺ iNKT cells reduce GVHD in a xenogeneic model of human cell transplantation

Irradiated NSG mice were transplanted with iNKT-depleted human PBMCs (PBMC alone group) or PBMC supplemented with either autologous ex vivo-expanded CD4⁻ iNKT (PBMC+iNKT4neg group) or CD4⁺ iNKT cells (PBMC+iNKT4pos group) at an iNKT/T cell ratio of 1:6. Co-administration of CD4⁺ iNKT cells and human PBMCs prolonged the overall survival (Figure 1A) and reduced weight loss (Figure 1B), clinical GVHD (Figure 1C) and liver pathological scores (Figure 1D) in comparison to mice transplanted with human PBMCs alone. In contrast, addition of CD4⁺ iNKT cells to PBMCs had no effect. As described in this model after low doses of PBMC injection, skin and gut clinical symptoms were invariably absent at 4 weeks after transplantation and pathological GVHD scores in these organs were very limited (Fig S1).

The reduction of xenogeneic GVHD in mice receiving human CD4⁺ iNKT cells was not due to impaired engraftment of human T cells, since kinetics and rates of CD45⁺ human cell engraftment in mouse peripheral blood were similar between all groups of mice (Figure 1E). At day 35, percentages of circulating human CD3⁺ T cells and CD4⁺ CD8 T cell ratios were similar in all groups (Figure 1F). Percentages of human T cells were also similar between all groups in the spleen and bone marrow (Fig S2). Of note, no circulating human iNKT cells were observed in any of the groups after day 21 of human cell transplantation.

Human CD4⁻ and CD4⁺ iNKT cells differentially modulate human T cell activation and cytokine responses in the context of xenogeneic activation in vivo

We observed that co-administration of iNKT cell subsets did not inhibit human T cell proliferation in vivo as evaluated by incorporation of BrdU (Figure 2A). However, in the PBMC+iNKT4pos group, there was reduced expression of the activation markers, CD25 and CD69, on circulating human CD4⁺ T and CD8⁺ T cells in comparison to the PBMC+iNKT4neg group (Figure 2B, 2C). We did not observe any significant differences in the proportion of FoxP3⁺ CD4⁺ T cells among total human CD4⁺ T cells in the blood, spleen and bone marrow between the 3 groups of mice (Figure 2D). Measurement of plasma levels of human cytokines in the plasma of the BMT recipients showed reduced levels of IL-17, IFN-γ, TNF-α and but increased concentrations of IL-4 at day 21 in the PBMC+iNKT4pos group in comparison to mice transplanted with PBMCs alone (Figure 2E). Intracellular staining of human CD4⁺ T cells isolated from the spleen of BMT recipients showed significantly lower percentages of IL-17 and RORγt⁺ cells in the PBMC+iNKT4pos group compared to the other groups (Figure 2G).

Human iNKT cell subsets have differential effects on mouse DCs in vitro and in vivo

Because antigenic presentation is a primary event in the T cell activation and cytokine response, we further analyzed the effects of CD4⁺ and CD4⁻ iNKT cells on mouse DCs in the model of xenogeneic GVHD. At day 28 after BMT, we observed reduced frequencies of CD11c⁺ murine DCs in the spleens of recipients in the PBMC+iNKT4pos group in comparison to the other groups (Figure 3A). To determine the maturation status of the CD11c⁺ population, we measured the expression of several markers associated with DC differentiation. We found that the expression of CD40, CD80 and CD86 were increased upon murine CD11c⁺ DCs in the PBMC+iNKT4pos group compared to human PBMCs alone (Figure 3B). In contrast, murine DC expression of CD40 and CD86 in the PBMC+iNKT4pos group was similar to mice transplanted human PBMCs alone (Figure 3B).

As human iNKT have been reported to recognize murine CD1d, we further analyzed whether human CD4⁺ or CD4⁻ iNKT cells could interact with NSG mouse (mo) BMDCs in vitro. Image stream analysis of co-cultures of human CD4⁺ and CD4⁻ iNKT cells with murine BM-derived DCs at a 2:2:1 ratio, showed that both CD4⁺ and CD4⁻ human iNKT subsets could form contacts with the murine cells (Figure 3C). However, human CD4⁻ iNKT cells made more than 3-fold more contacts than CD4⁺ iNKT cells (76.3 versus 23.7%, p < 0.0001) (Figure 3D).
suggesting that the former population could outcompete the latter for contacts with DC. To test whether increased contact formation was associated led to cytotoxic molecule degranulation, we determined the CD107a externalization. As shown in Figure 3E, CD107a externalization was significantly higher in CD4− compared to CD4+ iNKT cells, suggesting a higher degranulation capacity of iNKT CD4− cells in contact with murine DC.

**Human iNKT cell subsets have opposite effects on human monocyte-derived DCs in vitro**

Based on the distinct effects of human iNKT subsets observed on mouse BM-derived and splenic DCs, we further explored the effects of human iNKT subsets on the survival and maturation of human monocyte-derived DCs (moDCs) in vitro. Human CD4+ iNKT cells have been reported to be capable of killing human mature moDC at a high iNKT:DC ratios.16 We comparatively analyzed the capacity of both human CD4+ and CD4− iNKT cells to induce the apoptosis or necrosis of mature moDCs at different iNKT:DC ratios in vitro. We found that both iNKT cell subsets could kill mature moDCs in a dose-dependent manner from iNKT:DC ratios of 1:1 to 10:1, mainly by inducing the apoptosis of moDCs (Figure 4A). At iNKT:T ratios above 2:1, CD4− iNKT cells were consistently more efficient than CD4+ iNKT cells at inducing early (Annexin V+ PI−) and late (Annexin V+ PI+)...
Figure 2. In vivo assessment of mouse serum levels of human cytokine and T cell polarization after human PBMC + iNKT CD4− or CD4+ injection.

(A) BrdU staining, performed on splenic cells and assessed on human CD3+ (left panel) and CD4+ (right panel) T cells, showed similar levels of proliferating T cells between all groups on day 28 post-transplantation (p > 0.05 in all comparisons). (B) Expression of CD25 (upper panel) and CD69 (lower panel) on circulating human CD4+ T cells were reduced in mice transplanted with human PBMCs + CD4− iNKT cells (n = 15) as compared to those receiving PBMCs alone (n = 6) (p = 0.0069 and p = 0.0375, respectively) and PBMCs + CD4+ iNKT cells (n = 12) (0.0017 and 0.0038, respectively). (C) Expression of CD25 (upper panel) and CD69 (lower panel) on circulating human CD8+ T cells were reduced in mice transplanted with human PBMCs + CD4− iNKT cells (n = 15) as compared to those receiving PBMCs alone (n = 6) (p = 0.2065 and p = 0.0020, respectively) and PBMCs + CD4+ iNKT cells (n = 12) (0.0006 and 0.00158, respectively). (D) Shows comparable FoxP3 expression on human CD4+ T cells from blood, spleen and bone marrow of NSG mice transplanted with human PBMCs alone or with co-administration of CD4+ or CD4− iNKT cell. (E) Human cytokine measurement in plasma of NSG mice at day 21 after transplantation with human cells showed reduced levels of IL-17, TNF-α, INF-γ but increased concentrations of IL-4 in the group of mice transplanted with human PBMC and CD4− iNKT cells (n = 18) in comparison of those having received human PBMCs alone group (n = 8) (p ≤ 0.05 for all cytokines). Mice transplanted with human PBMCs and CD4+ iNKT cells (n = 22) had reduced plasma levels of INF-γ (p ≤ 0.05) and increased IL-4 (p ≤ 0.01) but similar IL-17 and TNF-α levels in comparison to control mice transplanted with human PBMCs alone. (F) Ratio of Th1+Th17/Th2 cytokines was significantly decreased in sera of mice injected with PBMC+CD4− iNKT compared to PBMC alone (p = 0.0397) (lower panel). (G) Intracellular staining of IL-17 and ROR-γt in peripheral blood T cells of NSG mice at day 21 after transplantation with human cells showed reduced levels of IL-17 and ROR-γt in the group of mice transplanted with human PBMC and CD4− iNKT cells (n = 6) in comparison of those having received human PBMCs alone group (n = 4) (p = 0.0293 and p = 0.0290 respectively). Data are pooled from 3 (A,B,F), 4 (C) or 7 (D,E) different experiments. (ns = non-significant; *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001).
apoptosis of moDCs (Figure 4A). The induction of apoptosis of human MoDCs by both iNKT cell subsets was contact dependent (Figure 4B).

In addition, to a previous description of differential effects of human CD4⁻ and CD4⁺ iNKT cells on human DC maturation and function, we observed that human CD4⁺ iNKT cells induce the
maturation of moDCs in a contact-dependent manner even in the absence of additional maturation cytokines, whereas human CD4+ iNKT cells had no effect (Figure 4C). The differential effect of CD4+ and CD4− iNKT cells on the maturation of immature MoDCs might be explained by higher expression of CD40-L on CD4+ in comparison to CD4− iNKT cells (Figure 4D).

Taken together, these data show that unlike their CD4+ counterparts, human CD4− iNKT cells show greater...
proficiency for inducing the apoptosis of mature DCs and do not induce the maturation of immature DCs.

Discussion

There in an increasing interest in using iNKT cells as a cellular therapy for GVHD control.\textsuperscript{33,34} In this study we aimed to identify which subtype of human iNKT might prevent GVHD and its mechanism of action in a xenogeneic GVHD model. We show that human CD4\textsuperscript{+} iNKT cells and not their CD4\textsuperscript{−} counterpart protect xenografted mice from GVHD by interfering with recipient APCs and human T cell responses. Human CD4\textsuperscript{−} iNKT cells have the unique potency to reduce the numbers and maturation levels of recipient APCs and therefore to reduce human allose- and xenogeneic T cell activation as well as Th1 and Th17 T cell cytokine production without inhibiting human T cell engraftment.

These results are in line with our and others’ recent reported data showing that CD4\textsuperscript{+} iNKT cell graft content and/or expansion capacities represent major predictive factors of the occurrence of acute GVHD in allografted patients.\textsuperscript{15,16,28} Nevertheless, the results obtained in humans appear in apparent contradiction with the description of a protective effect of murine CD4\textsuperscript{+} iNKT cells\textsuperscript{9,11,14,25} since IL-4 is mainly produced by CD4\textsuperscript{+} human iNKT cells. However, to our knowledge, no study has compared the differential effects of murine CD4\textsuperscript{−} versus CD4\textsuperscript{+} iNKT cell subsets, which seems puzzling as both mouse CD4\textsuperscript{+} and CD4\textsuperscript{−} iNKT cells can produce IL-4.\textsuperscript{22} The protective effect of human CD4\textsuperscript{−} iNKT cells, which are functionally characterized by the rapid production of high amounts of INF-γ, in the context of GVHD can also be conciled with the described benedic effect of INF-γ when administered early after allo-SCT.\textsuperscript{36} Moreover, in a mouse model of GVHD, infusion of murine type II CD8\textsuperscript{+} NK cells secreting high amounts of INF-γ has been reported to delay the occurrence of GVHD.\textsuperscript{37,38}

Another apparent discrepancy between murine and human iNKT cells in the regulation of GVHD is the description of interactions between iNKT cells and Tregs in mouse models of GVHD\textsuperscript{5,27,35} that has not been observed in our mouse model. This might be explained by the relatively limited numbers of Tregs injected within the 3 \times 10\textsuperscript{6} human PBMC graft and their absence in NSG mice. This might also be due to intrinsic limits of the xenogeneic GVHD model, which in the absence of production of human cytokines, such as IL-2, might be associated with impaired long-term survival of human Treg and iNKT cells. In favor of this hypothesis, we could not detect either Treg or iNKT in the peripheral blood, spleen or bone marrow of transplanted mice after engraftment of human T cells; we cannot, however, exclude their earlier migration to other tissues.

Mice receiving CD4\textsuperscript{−} iNKT cells had reduced expression of human T cell activation markers and of Th1 and Th17 cytokines in comparison to mice transplanted with or without CD4\textsuperscript{+} iNKT cells. Moreover, human iNKT have been reported to switch human DCs towards a tolerogenic phenotype.\textsuperscript{32} These facts led us to hypothesize differential modulatory effects of human CD4\textsuperscript{+} and CD4\textsuperscript{−} iNKT cell subsets on APCs during the allogeneic immune response. Whereas both subsets induced the apoptosis of mature monocyte-derived DCs, the human CD4\textsuperscript{−} iNKT subset had higher cytotoxic capacities. This observation is in agreement with reported higher expression of cytotoxic molecules such as NKG2D or perforin/granzyme by CD4\textsuperscript{−} in comparison to CD4\textsuperscript{+} iNKT cells.\textsuperscript{16,32} In vitro, by contrast with their CD4\textsuperscript{+} counterparts, CD4\textsuperscript{−} iNKT cells induce the maturation of monocyte-derived DCs without any additional cytokines, in a contact-dependent manner, possibly related to their higher expression of CD40-L than CD4\textsuperscript{+} iNKT cells.\textsuperscript{32} As human CD1d recognition by iNKT are largely conserved between species,\textsuperscript{30,31} we hypothesized that human iNKT could modulate mouse DC maturation and survival and explain by this way the modulation of xenogeneic GVHD. We indeed showed that human iNKT cells can interact with mouse DCs and that CD4\textsuperscript{−} iNKT cells are more likely to make contacts with mouse DCs and to kill murine DCs in vitro and in vivo than CD4\textsuperscript{−} iNKT cells. By contrast and compared to their CD4\textsuperscript{−} counterparts, CD4\textsuperscript{+} iNKT cells induce the maturation of immature DCs without impairing the numbers of splenic DCs in vivo. These opposite effects of CD4\textsuperscript{−} and CD4\textsuperscript{+} iNKT cells on DC maturation and survival can certainly explain the increased T cell activation and absence of protection from GVHD observed in the presence of CD4\textsuperscript{−} in comparison to CD4\textsuperscript{+} iNKT cells.

In conclusion, our data demonstrate that human CD4\textsuperscript{−} iNKT cells better control the in vivo human allogeneic T cell responses compared to their CD4\textsuperscript{+} counterparts. These data are of major importance to guide expansions protocols and cellular target therapy in the prevention of human acute GVHD.

Material and methods

Human iNKT expansion

Human iNKT were expanded from peripheral blood mononuclear cells (PBMC) from healthy volunteers (Etablissement Français du Sang (EFS), agreement N°14/EFS/018) cultured with alpha-galactosylceramide (α-GalCer) 100 ng/mL, (KRN7000, Avanti Polar Lipids, Alabaster, AL, USA) and rIL-2 (845 IU/mL, ImmunoTools, Friesoythe, Germany) for 15 days as described elsewhere.\textsuperscript{28}

Mice and xenogeneic mouse model of GVHD

NOD.Cg-PkdcsildIl2rgm1Wjl/Szl (NSG) mice, aged from 8 to 12 weeks, provided by Lucienne Chatenoud (INSERM U1013, Paris, France) were irradiated sub-lethally using 200-cGy total body irradiation by x-ray (RS 2000, Rad Source) on day –1, followed on day 0, by the intravenous infusion of 3 \times 10\textsuperscript{6} PBS57 tetramer negative PBMCs. Some mice were supplemented with ex
vivo expanded and flow sorted 2.5 × 10^5 CD4+ or CD4− iNKT cells from the same donor.

Mice were evaluated in a blinded fashion, twice a week, for survival and GVHD clinical score as reported elsewhere. According to our local Ethical Committee, mice were euthanized when achieving critical signs (weight loss above 20% and/or critical clinical signs).

In some experiments, 100 μL of peripheral blood was drawn weekly from the mandibular vein to assess engraftment of human cells. Plasma was stored at −80°C until analysis. All protocols were approved by the local Ethical Committee (CEEA34.0AP.018.11).

**Histology**

Pathological GVHD assessment was performed 4 weeks following human PBMC injection. Liver, small intestine and skin tissues were placed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin–eosin–safran (HES). Anti-hCD3 (eBioscience, Paris, France) staining was performed when appropriate. GVHD pathological scoring was performed under blinded conditions as described elsewhere. Photographs were taken using Å ~ 400 magnification with a Leica DF295 camera mounted on a Leica DMLB microscope.

**Generation of mouse bone marrow derived dendritic cells (MoBMDCs)**

Bone marrow cells of NSG mice were plated at a density of 1 × 10^6/mL in complete IMDM medium (10% FBS 50 IU/mL penicillin, 50 IU/mL streptomycin, 1% HEPES, 5 × 10^-5 M β-mercaptoethanol) supplemented with GM-CSF obtained from J558 hybridoma. After 3 days of culture, 75% of the media and non-adherent cells were removed and complete IMDM media supplemented with GM-CSF was added back. At day 7, all the media was gently removed, and adherent MoBMDCs were dislodged out of the culture plate by up and down pipetting with frozen 3mM EDTA in PBS. MoBMDC were assessed by FACS for their mCD11cexpression.

**Image-stream flow cytometry (AMNIS) and degranulation assay**

MoBMDCs (50 000 per well) stained with anti-mCD11c mAb were cultured with CD4+ iNKT or CD4+ iNKT sorted cells or a mixture of the two subsets (50% CD4+ and 50% CD4− iNKT) at a iNKT4+:iNKT4−:DC ratio of 2:2:1 in 100 μL of complete RPMI medium supplemented with anti-hCD107a-PE mAb (eBioscience, Paris, France) or the corresponding IgG1 isotype control and Monensin A. Cultures were performed in a 96 round bottom well plate. After 4 hours of culture, cells were fixed by ethanol free 4% PFA, washed and stained with Hoechst 33342 (Invitrogen, Life Technologies, Villebon sur Yvette, France). Cells were run on an Imagestream ISX mkII (Amnis Corp. Millipore, Seattle, WA, USA) and a 60X magnification was used for all acquisitions. Data were acquired using the INSPIRE software (Amnis Corp) and analyzed using the IDEAS software (version 6.2 Amnis Corp). Approximately 10,000 events were collected in all experiments.

**Cytokine concentrations**

The concentrations of human cytokines in mouse plasma were determined using a customized Bio-Plex Pro Human Cytokine 17-plex Assay (Bio-Rad Laboratories Inc, France). Results were analyzed on a Bio-Plex200 Instrument (Luminex xMap Technology).

**Flow cytometry and cell sorting**

For ex vivo mouse DC analysis, splenocytes were obtained by pressing the spleen through a 100μm strainer with 120U/mL DNase I treatment (Sigma-Aldrich, Saint-Quentin Fallavier, France). All ex vivo obtained cells were pre-incubated with CD16/CD32 mAb (clone 2.4G2) to block Fcγ receptors (eBioscience, Paris, France). Mouse CD11c+ DCs were analyzed in the human CD45− gate. For ex vivo intracellular cytokine staining, mice blood samples were first incubated in 1X Red cells Lysis buffer (BD Bioscience, Le Pont de Claix, France), and cells were stimulated for 4 h in complete RPMI medium (RPMI-1640 medium, 10% fetal bovine serum (FBS) 100 IU/mL penicillin, 100 IU/mL streptomycin, 200 mmol L-Glutamine, 10 mMol HEPES, all from Life Technologies, Villebon-sur-Yvette, France) in the presence of 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 500 ng/ml ionomycin and 10 μg brefeldin A (all from Sigma-Aldrich, Saint-Quentin Fallavier, France). Intracellular staining was performed on fixed/permeabilized cells (Fix/Perm solution, eBioscience, Paris, France) according to manufacturer’s instructions. Human cells were gated according to human CD45 staining.

Apoptosis was assessed using Propidium Iodide (PI) and Annexin V staining in 1X Annexin V buffer (eBioscience, Paris, France). BrdU staining was done on spleen cells according to manufacturer’s instructions (BrdU staining proliferation kit, BD Bioscience, Le Pont de Claix, France).

Monoclonal antibodies used for cell staining are listed in Table S1. Cells were analyzed on a FACS Canto II (BD Biosciences, Le Pont de Claix, France), coupled with FlowJo software vX.0.7 (Tree Star Inc. San Diego, CA, USA). Cell sorting was performed on BD FACS Aria (BD Bioscience, Le Pont de Claix, France).

**Generation of human monocyte-derived DC**

Monocytes were isolated from human PBMC by CD14 positive magnetic cell sorting (Miltenyi Biotec Monocyte Isolation Kit I, Miltenyi Biotec SAS, Paris, France). Immature DC were obtained by culturing monocytes in supplemented complete RPMI medium in the presence of GM-CSF (1000 IU/ml) and IL-4 (500 IU/ml, all from RD Systems, Lille, France) for 6 days. Mature DC were obtained by addition of 10 ng/ml prostaglandine E2 (Sigma-Aldrich, Saint-Quentin Fallavier, France) + 1000 UI/mL TNFa (RD Systems, Lille, France) for 24 hours.

**iNKT-DC cultures**

In the maturation assays, unrelated CD4+ or CD4− iNKT were cultured at 1:1 ratio with immature DCs, in contact or in transwell chambers (Costar, NY). In the apoptosis assays,
unrelated iNKT CD4⁺ or CD4⁻ were added to mature DCs at various iNKT/DC ratios. PI and Annexin V staining was performed after 4 to 24 hours of co-culture according to manufacturer’s instructions (eBioscience, Paris, France). DCs were analyzed in the PBS57 tetramer negative gate.

**Statistical analysis**

Comparison tests were performed using analysis of variance for repeated measurements followed by Student’s t test or Fisher’s exact test when appropriate. Survival curves were compared by log-rank test. The results are expressed as the mean ± SEM. The p-values were identified as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Statistical analyses were performed using Prism version 5.01 (GraphPad software).

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**Authorship contributions**

TC, MTR, OH designed the experiments, interpreted the data and wrote the manuscript.

TC, JR and MD performed the experiments.

MD performed the confocal analyses.

BF performed the histopathology analyses.

RR and MB provided technical support.

JB, FC and IM provided a critical review of the data and manuscript.

All authors approved the manuscript.

**Disclosures of Conflicts of interests**

The authors declare no financial conflict of interest. Supplementary materials for this article can be accessed here.

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