ARTICLE

Human invariant natural killer T cells promote tolerance by preferential apoptosis induction of conventional dendritic cells

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ABSTRACT

Graft-*versus*-host disease (GvHD) is a major cause of morbidity and mortality after allogeneic hematopoietic cell transplantation. We recently showed in murine studies and *in vitro* human models that adoptively transferred invariant natural killer T (iNKT) cells protect from GvHD and promote graft-*versus*-leukemia effects. The cellular mechanisms underlying GvHD prevention by iNKT cells in humans, however, remain unknown. In order to study relevant cellular interactions, dendritic cells (DC) were either generated from monocytes or isolated directly from blood of healthy donors or GvHD patients and co-cultured in a mixed lymphocyte reaction (MLR) with T cells obtained from healthy donors or transplantation bags. Addition of culture-expanded iNKT cells to the MLR-induced DC apoptosis in a cell contact-dependent manner, thereby preventing T-cell activation and proliferation. Annexin V/propidium iodide staining and image stream assays showed that $CD4^+CD8^-$, $CD4^-CD8^+$ and double negative iNKT cells are similarly able to induce DC apoptosis. Further MLR assays revealed that conventional DC (cDC) but not plasmacytoid DC (pDC) could induce alloreactive T-cell activation and proliferation. Interestingly, cDC were also more susceptible to apoptosis induced by iNKT cells, which correlates with their higher CD1d expression, leading to a bias in favor of pDC. Remarkably, these results could also be observed in GvHD patients. We propose a new mechanism how *ex vivo* expanded human iNKT cells prevent alloreactivity of T cells. iNKT cells modulate T-cell responses by selective apoptosis of DC subsets, resulting in suppression of T-cell activation and proliferation while enabling beneficial immune responses through pDC.

Introduction

Despite significant advances in the field of allogeneic hematopoietic cell transplantation (HCT), graft-*versus*-host disease (GvHD) still represents a major complication after allogeneic HCT, leading to substantial morbidity and mortality.[1,2] GvHD is mediated by donor T cells activated through antigen-presenting cells (APC).[3] Dendritic cells (DC) are professional APC that precisely orchestrate adaptive immune responses and their significant role in GvHD pathophysiology has been established previously.[4-6] Both donor and host DC present host antigens and promote activation and proliferation of alloreactive donor T cells, which consequently home to GvHD target sites, resulting in tissue destruction and clinical manifestations of GvHD.[7,8] The ability of DC to elicit or prevent T-cell responses is tuned by the concomitant expression of stimulatory or inhibitory molecules as well as immunomodulatory cytokines.[9]

DC also express antigen-presenting molecules such as the major histocompatibility complex-I (MHC-I)-like molecule CD1d that allows for interactions with invariant natural killer T (iNKT) cells. iNKT cells are a small subset of T lymphocytes characterized by the expression of an invariant T-cell receptor in both humans and

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mice.[10] Upon activation through glycolipids, iNKT cells regulate immune responses by the instant release of immunoregulatory cytokines or by direct cell killing.[11-13]

Several studies have shown the ability of iNKT cells to reduce the incidence of GvHD. In murine models, iNKT cells prevent acute and chronic GvHD, while promoting beneficial graft-*versus*-leukemia (GvL) effects.[14-16] In humans, clinical studies have demonstrated that high iNKT-cell numbers are associated with a diminished occurrence of GvHD.[17-19] Moreover, we recently showed that culture-expanded human iNKT cells are able to prevent T-cell activation and proliferation while exerting potent anti-leukemic activity.[13,20]

Nevertheless, the complex cellular and molecular mechanisms of immune tolerance induction through iNKT cells remain poorly understood. In this study, we focused on how culture-expanded human iNKT cells modulate alloreactive T-cell responses through DC in healthy volunteers and GvHD patients.

Methods

Research subjects

Human buffy coats from healthy volunteers were obtained from the Center of Clinical Transfusion Medicine Tuebingen. Samples from hematopoietic cell grafts and peripheral blood mononuclear cells (PBMC) from patients with GvHD were isolated after written informed consent had been obtained. Human leukocyte antigen (HLA) typing was performed by the Center of Clinical Transfusion Medicine Tuebingen or the HLA laboratory of the Department of Medicine II of the University Hospital Tuebingen. The study was approved by our Institutional Review Board to be in accordance with ethical standards and with the Helsinki Declaration of 1975, as revised in 2013 (IRB approvals 483/2015BO2 and 137/2017BO2).

Flow cytometry

Antibodies and reagents used for flow cytometric analyses are described in the *Online Supplementary Appendix*.

Invariant natural killer T-cell expansion and enrichment

iNKT cells were expanded from third-party PBMC with some minor modifications as previously described (*Online Supplementary Appendix*).[13] Culture-expanded iNKT cells were purified with anti-iNKT MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Alternatively, iNKT cells were stained with DAPI (4',6-diamidino-2-phenylindole, Merck, Darmstadt, Germany), anti-CD3, anti-CD4, anti-CD8 antibodies and PBS57-loaded CD1d tetramer allowing for enrichment of iNKT cells and their different subsets by fluorescence-activated cell sorting (FACS).

Generation of monocyte-derived dentritic cells and isolation of blood dendritic cells

Monocyte-derived dentritic cells (Mo-DC) were generated as described previously.[13] Blood DC from healthy donors and patients were isolated using Blood Dendritic Cell Isolation Kit II (Miltenyi Biotech). Where indicated, HLA-DR$^+$ blood DC were further sorted either as CD1c$^+$ conventional DC (cDC) or CD303$^+$ plasmacytoid DC (pDC).

CD3$^+$ T-cell isolation

CD3$^+$ T cells were isolated from human PBMC with anti-CD3 MicroBeads (Miltenyi Biotech). For proliferation analysis, T cells were marked with CFSE (carboxyfluorescein succinimidyl ester, Biolegend, San Diego, CA, USA) according to the manufacturer's instructions and tested in a mixed lymphocyte reaction (MLR).

Mixed lymphocyte reaction

Major mismatched mo-DC or blood DC were plated together with allogeneic CD3$^+$ T cells at a 1:1 ratio. Culture-expanded iNKT cells were added to the MLR at different doses, either directly or separated from the MLR by 0.4 µm TC-Inserts (Sarstedt, Nuembrecht, Germany). Cells were analyzed by flow cytometry for activation markers (CD69 and CD25) and proliferation (CFSE). Alternatively, T cells were incubated with anti-CD3/CD28-coated beads (ThermoFisher Scientific, Waltham, MA, USA) in the presence or absence of iNKT cells. For blocking assays, iNKT cells or DC were pre-treated with the respective antibodies or IgG control (*Online Supplementary Appendix*).

Apoptosis assays

Apoptosis was assessed with an annexin V-FITC/propidium iodide (PI) Staining Kit (BD Bioscience, Franklin Lakes, NJ, USA), by cell cycle analysis modified according to Nicoletti[21] or by image stream analysis (*Online Supplementary Appendix*). The percentage of apoptotic cells was determined by flow cytometry.

Cytokine analysis

Cell culture supernatants from MLR were collected after 4 and 24 hours (h), respectively. In order to analyze cytokine production bead-based immunoassays were performed according to the manufacturer's instructions. Cytokine release was measured by a LEGENDplex human CD8/NK-cell panel (BioLegend). Data were acquired using the Lyric flow cytometer with autosampler (BD Biosciences).

Statistical analysis

Student's *t*-test and analysis of variance (ANOVA) were used for statistical analysis. $P<0.05$ was considered statistically significant. Data were analyzed with Prism 8 (GraphPad Software, La Jolla, CA, USA). All experiments were performed at least in technical duplicates and repeated independently at least three times using different iNKT-cell donors.

Results

Invariant natural killer T cells inhibit T-cell activation and proliferation in a cell contact-dependent manner

Human culture-expanded iNKT cells suppress alloreactive T-cell responses when T lymphocytes are stimulated by MHC-mismatched DC.[13] As iNKT cells exhibit potent immunoregulatory properties through a rapid release of humoral mediators, we wondered whether this effect might be related to the inhibition of T-cell function.[22] Therefore, we measured early (CD69 expression) and late activation (CD25 expression) as well as proliferation (CFSE dilution) of T cells co-incubated with DC in presence or absence of iNKT cells. iNKT cells were either added directly to the MLR or separated through a TC-insert (transwell [TW]). Direct addition of iNKT cells at different ratios to the MLR reduced T-cell activation and proliferation in a dose-dependent manner. However, iNKT cells which were separated by a TW did not prevent T-cell activation and proliferation (Figure 1A to C; *Online Supplementary Figure S1*). We conclude from these experiments that iNKT cells mostly rely on direct cell contact to efficiently suppress alloreactive T-cell responses. In addition, T-cell activation and proliferation initiated by artifi-

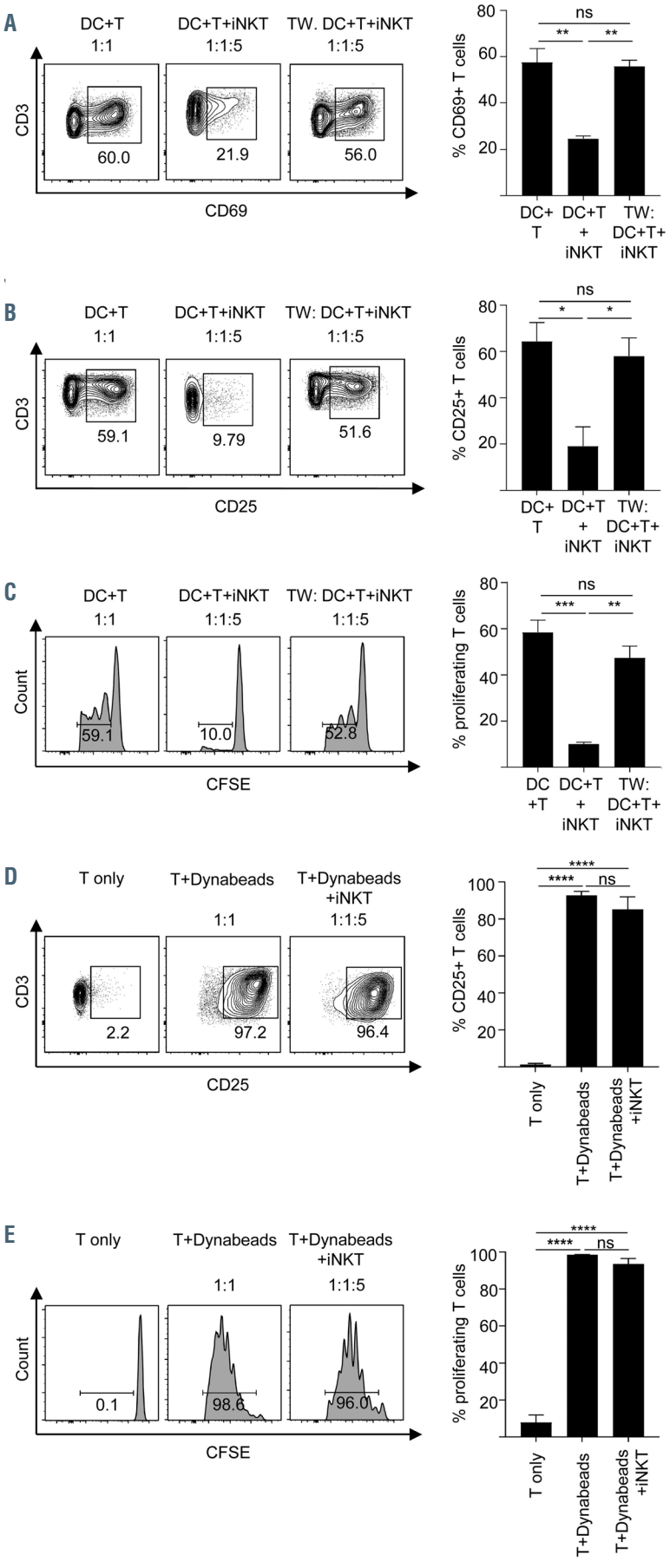

Figure 1. Culture-expanded invariant natural killer T cells inhibit T-cell activation and proliferation. Representative dot plots and histograms showing (A) early activated T cells (CD69$^+$, day 1), (B) late activated T cells (CD25$^+$, day 3) and (C) proliferating T cells (carboxyfluorescein succinimidyl ester [CFSE], day 7). T-cell activation and proliferation was measured after incubation with monocyte-derived dendritic cell (mo-DC) in the presence or absence of invariant natural killer T (iNKT) cells. iNKT cells were added to the culture either directly or separately through a transwell insert (TW). (D) Representative dot plots and histograms showing late activated T cells (CD25$^+$, day 3) and (E) proliferating T cells (CFSE, day 7) after stimulation with anti-CD3/CD28-coated beads in the presence or absence of iNKT cells. All events were gated on single cells and living lymphocytes. iNKT cells were excluded from the analysis by gating on CD3+ PBS57-loaded CD1d tetramer$^+$ populations. Histograms show the mean of three independent experiments (n=3). Error bars indicate standard error of the mean. ns: not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. DC: dendritic cells; T: T cells.

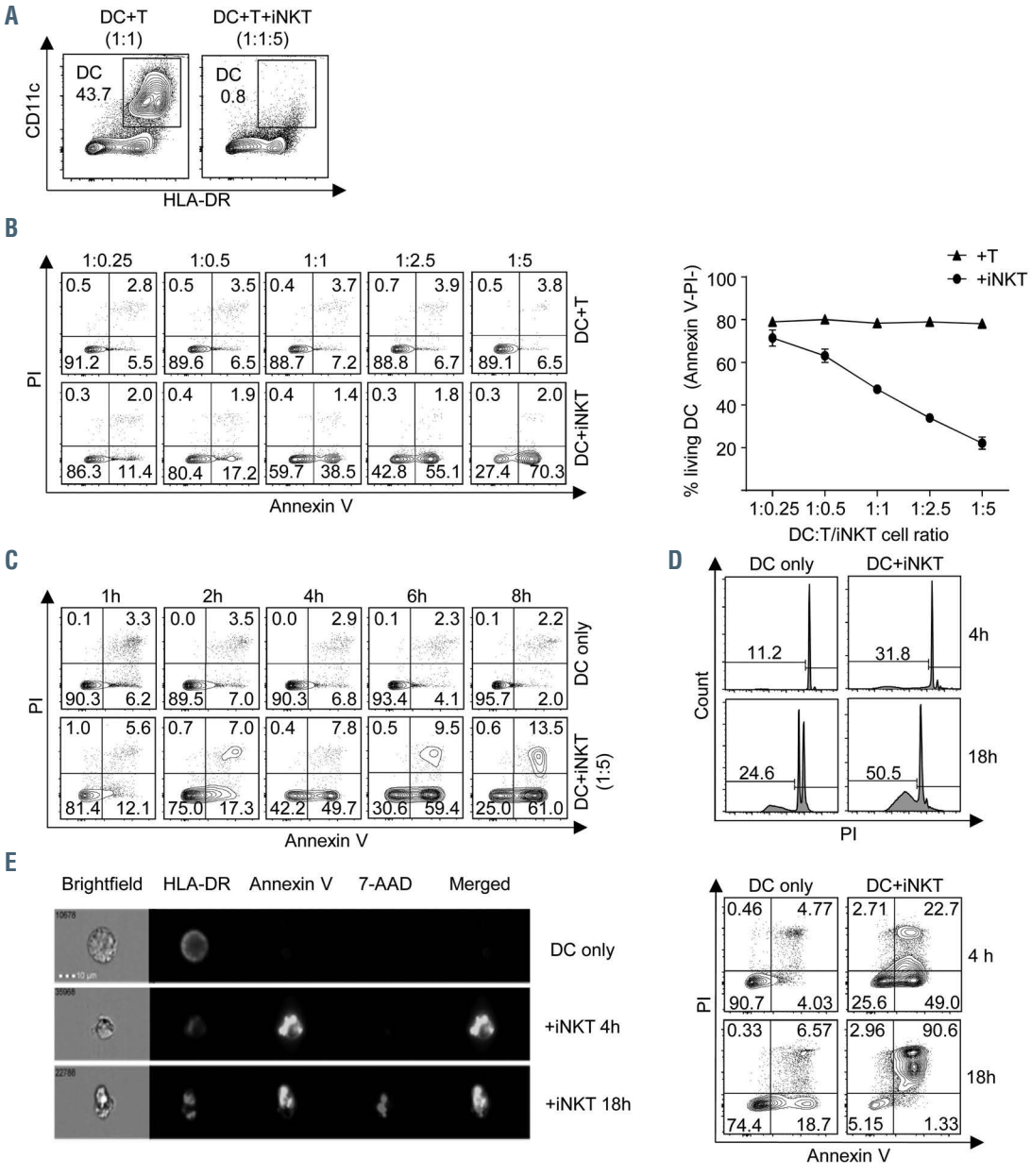

Figure 2. Culture-expanded invariant natural killer T cells induce dendritic cell apoptosis in a dose-dependent manner. (A) Representative dot plots showing absence of dendritic cells (DC) ($CD11c^+HLA\text{-}DR^+$) after co-culture with invariant natural killer T (iNKT) cells. (B) Representative dot plots and pooled data of living DC (annexin V-/propidium iodide [PI]-) after 4 hours (h) of co-culture with increasing numbers of T cells or iNKT cells. Indicated are the ratios of DC to T or iNKT cells. (C) Representative dot plots showing DC apoptosis in co-cultures with iNKT cells after 1, 2, 4, 6 and 8 h of incubation. (D) Histograms showing increased DNA fragmentation in DC after 4 h and 18 h of co-culture with iNKT cells. The gates on the left of each plot show the percentage of apoptotic nuclei. (E) Representative image stream assay and dot plots showing DC apoptosis induced by iNKT cells after 4 h and 18 h (annexin V^+, green; 7-AAD^+, red). (F) Representative dot plots, relative and absolute numbers of living DC (annexin V-/PI-) after 4 h of co-culture with different iNKT-cell subsets. Histograms show the mean of three independent experiments (n=3). Error bars indicate standard error of the mean. ns: not significant. HLA-DR: human leukocyte antigen DR-isotype.

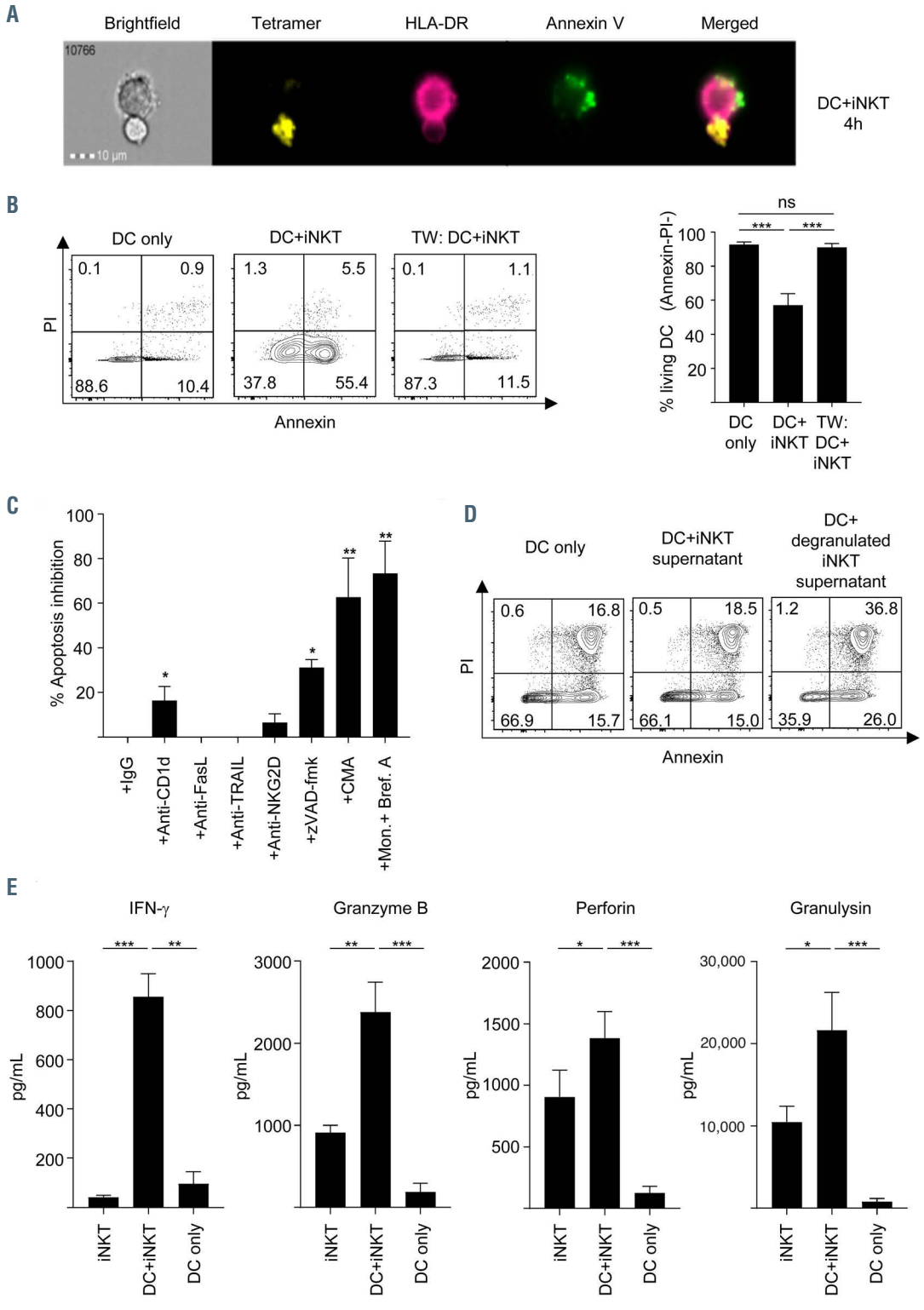

Figure 3. Culture-expanded invariant natural killer T cells require cell contact to induce apoptosis through degranulated effector molecules. (A) Representative image stream assay illustrating direct cellular contact between invariant natural killer T (iNKT) cells (PBS57-loaded CD1d tetramer$^+$, yellow) and dendrtic cells (DC) (HLA-DR$^+$, pink) and subsequent DC apoptosis induction (annexin V$^+$, green) after 4 hours (h) of co-incubation. (B) Representative dot plots showing DC apoptosis and pooled data of living DC (annexin V-propidium iodide [PI]-) after co-incubation with iNKT cells either directly or separated by a transwell insert (TW). (C) Percentage of DC apoptosis inhibition after blocking of the receptors CD1d, FasL, TRAIL, NKG2D and applying the inhibitors zVAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone), CMA (concanamycin A) and monensin/brefeldin A. (D) Representative dot plots showing DC apoptosis after co-culture with non-degranulated and degranulated iNKT-cell supernatant. (E) IFN-γ, granzyme B, perforin and granulysin release by iNKT cells after encountering DC analyzed by bead-based immunoassay. Histograms show the mean of three independent experiments (n=3). Error bars show standard error of the mean. ns: not significant; *P<0.05, **P<0.01, ***P<0.001; HLA-DR: human leukocyte antigen DR-isotype.

cial antigen-presenting cells (aAPC, e.g., Dynabeads) was only affected when higher numbers of iNKT cells were added to the culture (Figure 1D and E; *Online Supplementary Figure S2*). In particular, proliferation speed was decreased with a predominance of early daughter generations (*Online Supplementary Figure S2*). Our findings suggest that the interaction of iNKT cells with DC largely contributes to the control of alloreactive T cells although a minor direct impact of iNKT cells on T cells could be observed.

Invariant natural killer T cells induce apoptosis of allogeneic dendritic cells in a dose-dependent manner

We performed flow cytometry to determine the phenotype of DC challenged with iNKT cells. Notably, DC numbers were highly reduced (Figure 2A) and we suspected induction of apoptosis through iNKT cells. Annexin V assays showed that culture-expanded iNKT cells rapidly induced apoptosis of allogeneic DC, while co-culture of DC with conventional allogeneic $CD3^+$ T cells did not, suggesting that apoptosis induction is not only dose-dependent but also specific to iNKT cells (Figure 2B and C). The Nicoletti assay revealed that DC start to defragment their DNA after co-culture with iNKT cells, which represents a further hallmark of apoptosis (Figure 2D). Interestingly, an increase of spontaneous DNA defragmentation could be observed in DC without iNKT cells after 18 h which could be explained by the lack of specific stimuli. Image stream analysis also confirmed morphologic changes in DC after co-culture with iNKT cells. Whereas DC cultured alone presented a healthy and round morphology, DC co-cultured with iNKT cells were small, squashed and with a blobbing membrane. Further, upregulation of Annexin V and loss of nuclear integrity in DC co-incubated with iNKT cells could be observed in image stream assays, confirming our previous assumption (Figure 2E). Next, we investigated whether the induction of apoptosis is specific to certain iNKT-cell subpopulations. Therefore, culture-expanded iNKT cells were sorted into double negative, $CD4^+CD8^-$ and $CD4^-CD8^+$ subsets and co-cultured separately with allogeneic DC: all iNKT-cell subsets were able to induce apoptosis of DC with comparable efficiency (Figure 2F).

Induction of dendritic cell apoptosis is cell contact-dependent and mediated by cytotoxic effector molecules

In order to further elucidate the cellular and molecular mechanisms responsible for iNKT-cell-induced DC apoptosis, we first analyzed image stream data visualizing doublets consisting of DC and iNKT cells. Image stream analysis revealed a direct binding of iNKT cells (PBS57-loaded CD1d tetramer$^+$) to the surface of allogeneic DC ($HLA\text{-}DR^+$), which subsequently revealed positive surface staining for the apoptosis marker annexin V (Figure 3A). In order to test whether this direct cellular interaction is required, iNKT cells and DC were separated by a transwell insert demonstrating that iNKT cells were unable to induce DC apoptosis anymore (Figure 3B). We further performed blocking experiments of common key molecules to identify critical pathways responsible for apoptosis induction through iNKT cells. It was observed that blocking FasL, TRAIL or NKG2D did not significantly reduce apoptosis of DC exposed to iNKT cells. However, blocking the CD1d and invariant T-cell receptor interaction reduced DC apoptosis significantly indicating that T-cell receptor engagement contributes to efficient lysis. Further, blocking apoptosis via caspase inhibitor zVAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone) or inhibition of the perforin pathway via CMA (concanamycin A) also diminished iNKT-cell-mediated cell death of DC. Moreover, the inhibition of iNKT-cell degranulation by monensin and brefeldin A was shown to impede apoptosis induction most efficiently (Figure 3C; *Online Supplementary Figure S3*). By adding supernatant of DC-triggered degranulated iNKT cells to viable DC, we could show that iNKT cells released cytotoxic factors during degranulation which further induced apoptosis in DC (Figure 3D). In order to identify these factors, we performed bead-based multiplex assays and thereby revealed the release of interferon-γ (IFN-γ), granzyme B, perforin and granulysin (Figure 3E).

Invariant natural killer T cells induce preferential apoptosis of blood conventional dendritic cells in healthy donors and graft-*versus*-host disease patients

Our previous observations are based on *ex vivo* cultured mo-DC. In order to support our findings, we additionally performed MLR and apoptosis assays using blood DC isolated from PBMC of healthy donors and GvHD patients following allogeneic HCT. Blood DC are mainly composed of cDC and pDC with the latter expressing lower levels of CD1d (*Online Supplementary Figure S4A*). Also, human blood DC of healthy volunteers induce activation and proliferation of MHC-mismatched T cells that can be diminished through the addition of iNKT cells (Figure 4A). Given that iNKT cells interact with DC through CD1d and CD1d engagement contributes to efficient lysis of target cells, we aimed to determine how human blood pDC and cDC are susceptible to iNKT-cell apoptosis induction. For this purpose, we isolated HLA-DR+ pDC ($CD303^+$) and cDC ($CD1c^+$) by FACS, co-cultured them separately with iNKT cells for 4 h and stained with annexin V and PI. We observed preferential apoptosis induction of cDC, while pDC were less affected by the addition of iNKT cells (Figure 4B). Further, we wondered whether preferential apoptosis of cDC by iNKT cells would also affect T-cell alloreactivity using fresh human blood DC as stimulators. We observed that only allogeneic cDC in contrast to pDC could induce significant T-cell activation and proliferation (*Online Supplementary Figure S4B*). Consequently, co-culture of these distinct blood DC subsets with allogeneic T cells and iNKT cells revealed that iNKT cells were also able to suppress activation and proliferation of alloreactive T cells induced by cDC (Figure 4C).

Finally, we tested whether our findings also apply to patients with acute GvHD having received grafts from HLA-matched donors. Therefore, we isolated blood DC from PBMC obtained from patients with clinical manifestations of acute GvHD grade ≥2 prior to induction of systemic treatment with steroids. At the time point of blood collection patients had complete donor chimerism in their peripheral blood. cDC from GvHD patients also showed higher expression levels of CD1d (*Online Supplementary Figure S4C*) and were more susceptible to iNKT-cell-induced apoptosis than pDC (Figure 4D), similarly as demonstrated in our previous experiments with cells from healthy donors. Next, T cells derived from donors prior to transplantation were co-cultured with blood DC from GvHD patients. Importantly, adding iNKT cells from

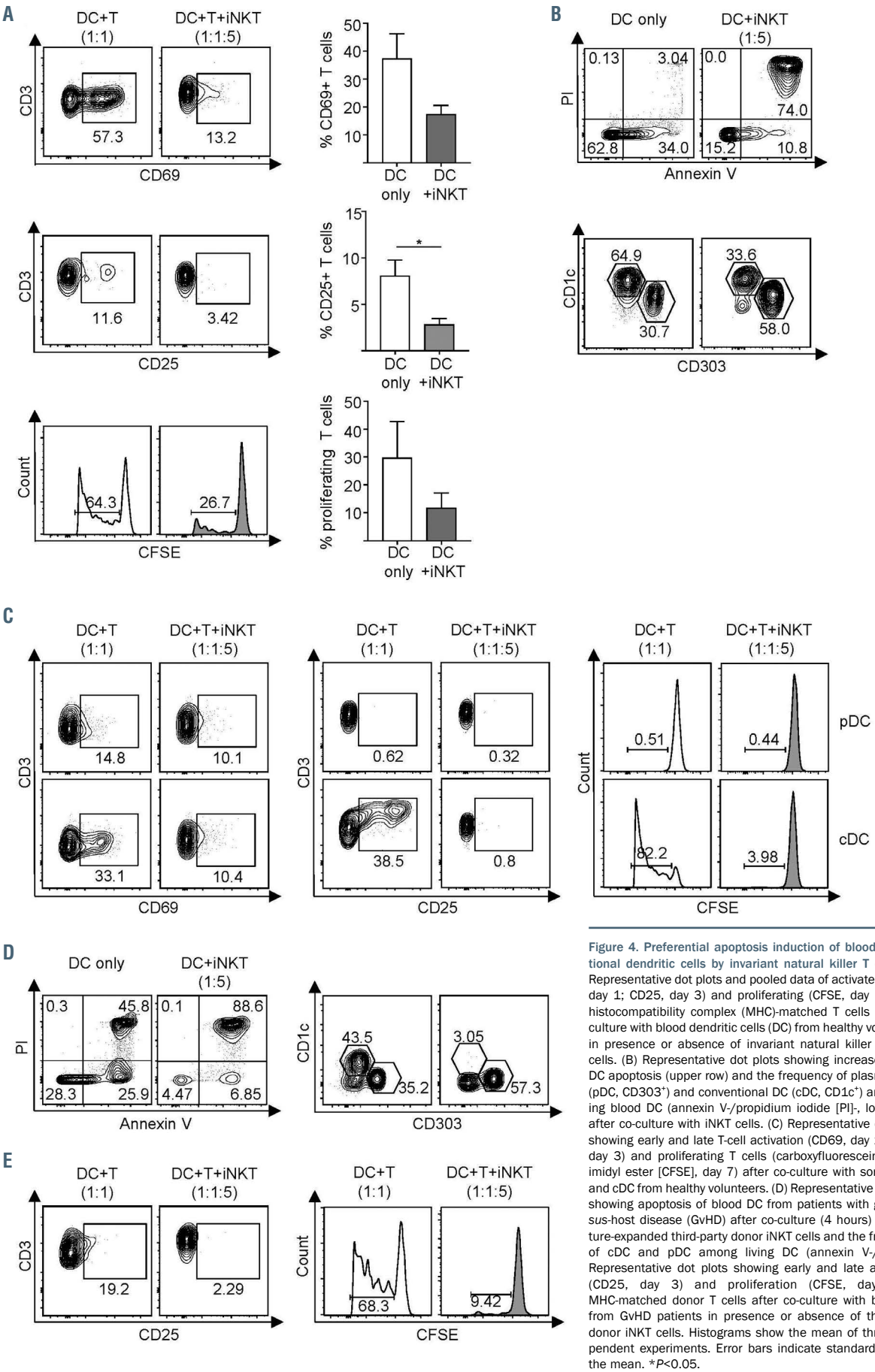

Figure 4. Preferential apoptosis induction of blood conventional dendritic cells by invariant natural killer T cells. (A) Representative dot plots and pooled data of activated (CD69, day 1; CD25, day 3) and proliferating (CFSE, day 7) major histocompatibility complex (MHC)-matched T cells after co-culture with blood dendritic cells (DC) from healthy volunteers in presence or absence of invariant natural killer T (iNKT) cells. (B) Representative dot plots showing increased blood DC apoptosis (upper row) and the frequency of plasmacytoid (pDC, $CD303^+$) and conventional DC (cDC, $CD1c^+$) among living blood DC (annexin V-/propidium iodide [PI]-, lower row) after co-culture with iNKT cells. (C) Representative dot plots showing early and late T-cell activation (CD69, day 1; CD25, day 3) and proliferating T cells (carboxyfluorescein succinimidyl ester [CFSE], day 7) after co-culture with sorted pDC and cDC from healthy volunteers. (D) Representative dot plots showing apoptosis of blood DC from patients with graft-*versus*-host disease (GvHD) after co-culture (4 hours) with culture-expanded third-party donor iNKT cells and the frequency of cDC and pDC among living DC (annexin V-/PI-). (E) Representative dot plots showing early and late activation (CD25, day 3) and proliferation (CFSE, day 7) of MHC-matched donor T cells after co-culture with blood DC from GvHD patients in presence or absence of third-party donor iNKT cells. Histograms show the mean of three independent experiments. Error bars indicate standard error of the mean. *P<0.05.

third-party donors also inhibited alloreactive T-cell activation and proliferation (Figure 4E).

Discussion

Allogeneic HCT is an established therapeutic option for the treatment of advanced and high-risk hematologic malignancies. Efforts to optimize donor selection, tailored preparative conditioning regimes and advanced supportive care have significantly contributed to improved outcomes and enabled long-term survival even in aged and comorbid patient populations. Nevertheless, GvHD and relapse still represent the most important reasons for significant morbidity and mortality after allogeneic HCT.[1,2] Various strategies have been applied to prevent or treat GvHD such as immunosuppressive medications and *in vivo* or *ex vivo* donor T-cell depletion. However, these approaches are suboptimal since they also inhibit immune reconstitution, pathogen control and beneficial GvL effects, leading to higher relapse rates.[23,34] Therefore, strategies that prevent GvHD while preserving the capacity of the graft to promote GvL effects are urgently needed.

A convincing body of evidence has demonstrated the potential of iNKT cells as a promising alternative for the prevention of GvHD in both mice and humans. Early murine studies demonstrated that the reinfusion of $NK1.1^+$ T cells after transplantation resulted in GvHD suppression.[25] In particular, low doses of $CD4^+$ iNKT cells prevented GvHD lethality in mice by promoting the expansion of Tregs while maintaining GvL effects.[14] We also showed previously that third-party iNKT cells are equipotent due to the highly conserved invariant TCR of iNKT cells.[15,26] Based on these findings, we used iNKT cells from third-party donors in our present study. In humans, several groups have shown that high numbers of iNKT cells were associated with a decreased incidence of GvHD.[17,18,27] Malard *et al*. also showed in a study of 80 patients that high iNKT-cell numbers in the graft correlated with an increased GvHD-free, relapse-free survival; however, the frequency of Tregs did not seem to correlate with iNKT-cell numbers.[19] Moreover, Cheng *et al*. analyzed Treg expansion after infusion of α-galactosylceramide (α-GalCer), a potent iNKT-cell stimulator, but expansion of Tregs could only be observed in a subset of patients.[28] Thus, the role of Treg expansion as mediator of therapeutically used iNKT cells is not well established in humans and suggests further mechanisms that contribute to the immunoregulatory properties of iNKT cells.

GvHD can be characterized as a response of donor T cells to host antigens presented by MHC molecules through APC1: first, host APC become activated and present allo-antigens to donor T cells, which are stimulated and expand. Consequently, cellular effectors promote cell damage and apoptosis.[1,8,29] In this context, several studies have emphasized the role of DC as potent APC in the pathogenesis of GvHD and therefore, they represent an interesting target for prophylactic and therapeutic strategies against GvHD.[30,31] In the present study, we therefore focused on the cellular and humoral interplay of human culture-expanded iNKT cells with DC. Thereby, we could show that iNKT cells induce DC apoptosis and consequently, impair alloreactive T-cell activation and proliferation. Liu and Coman reported similar findings previously hypothesizing a relevant mechanism for the modulation of immune responses and GvHD suppression.[32,33] We add significant knowledge by showing that preferential apoptosis induction of cDC leads to a relative expansion of beneficial pDC. In contrast, we did not find significant functional differences regarding distinct iNKT-cell subsets.

iNKT cells are activated upon recognition of glycolipids presented by the MHC-I-like molecule CD1d, which is highly expressed on DC.[11,34] Hence, T-cell receptor-CD1d engagement induces cytokine release by iNKT cells, which confers immunoregulatory properties and the ability to orchestrate immune responses of several cell types. For instance, the release of cytokines such as IFN-γ, tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-17 and IL-21 has been noted.[13,22] Beyond immunoregulatory properties, iNKT cells exert potent direct cytotoxic effects using different pathways.[13,20] In this context, stimulation via CD95 (Fas)[35,36] and TRAIL pathways[36,37] has been demonstrated, resulting in a classical lymphocytotoxic response against tumor cells. Moreover, several studies have shown that perforin/granzyme B is involved in iNKT-cell tumor cytotoxicity.[38-40] Using different blocking reagents and specific antibodies, we could demonstrate that DC apoptosis induced by iNKT cells relies on degranulation of perforin, granzyme B and granulysin and partially on the interaction of the invariant T-cell receptor with CD1d .

DC originate from either myeloid or lymphoid hematopoietic stem cell progenitors in the bone marrow.[41,42] They constitute a heterogeneous cell group of different subsets playing distinct roles in regulating immune responses.[43] DC have been categorized in cDC, pDC and mo-DC, considering their lineage and expression of transcription factors such as IFN regulatory factors 8 and 4.[44] In humans, cDC are potent producers of IL-12 and harbor excellent cross-priming properties. In the context of GvHD, cDC turned out to be important stimulators of alloreactive T-cell responses.[45] Also, Markley *et al*. demonstrated that donor cDC are critical for allo-antigen presentation and consequently potentiate GvHD.[46] Besides, cDC are most likely responsible for the replenishment of tissue-specific DC such as migratory Langerhans cells of the skin after inflammation and therefore might contribute to the occurrence and perpetuation of skin GvHD.[47,48] In contrast, the functional hallmark of pDC is the release of high quantities of type I and type III interferon (IFN) in response to viral antigen recognition.[49,50] Interestingly, precursor and fully differentiated pDC are associated with an improved outcome after allogeneic HCT due to a decreased incidence of GvHD and optimized GvL effects.[51,52] Thus, the modulation of cDC and pDC by iNKT cells could represent a useful approach to reduce the incidence of GvHD.

In this study, we focused on human blood DC, which are mainly composed of cDC and pDC and can be easily obtained from healthy volunteers and GvHD patients. Our results suggest an additional mechanism of how human culture-expanded iNKT cells prevent GvHD: preferential apoptosis of cDC leads to a relative expansion of beneficial pDC. This bias results in decreased activation and proliferation of alloreactive T cells from healthy volunteers and GvHD patients. However, we could also observe a minor direct impact of iNKT cells on T- cell activation and proliferation when higher numbers of iNKT cells were used. Given the high plasticity and functional

diversity of iNKT cells we assume that several mechanisms, that are not mutually exclusive, are generally involved in tolerance induction: modulation of DC function, expansion of FoxP3 regulatory T cells, induction of a Th2 bias of T-helper cells and decreased expansion of alloreactive donor T cells. Indeed, it has been observed that distinct iNKT-cell subsets are associated with certain functional properties which might explain different findings from other groups in humans and mice. Also, culture conditions might affect the function of iNKT cells after expansion.

In conclusion, we postulate an additional mechanism by which iNKT cells prevent GvHD in humans, focusing on their interaction with different DC subsets. iNKT cells promote selective cDC apoptosis through the release of effector molecules such as perforin and granzyme B in a cell-contact-dependent manner, which could consequently prevent GvHD. However, pDC are spared and may still convey beneficial immune responses leading to efficient GvL effects and pathogen control resulting in improved survival after allogenic HCT.

Disclosures

No conflicts of interest to disclose

Contributions

HS, EMR and DS designed and performed the research and analyzed data; K-AS SD-S, HK, RD, TM, KS-O, SH and CS performed the research and analyzed data; EMR and DS wrote the manuscript. All authors edited the manuscript for content.

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Data sharing statement

Raw data and detailed protocols of the used methods used can be obtained upon direct request to the corresponding author.

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