Activated iNKT cells promote Vγ9Vδ2-T cell anti-tumor effector functions through the production of TNF-α

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Abstract

Vγ9Vδ2-T cells constitute a proinflammatory lymphocyte subpopulation with established antitumor activity. Phosphoantigens activate Vγ9Vδ2-T cells *in vivo* and *in vitro*. We studied whether the antitumor activity of Vγ9Vδ2-T cells can be potentiated by invariant NKT cells (iNKT), an important immunoregulatory T cell subset. When activated by the glycolipid α-galactosylceramide (α-GalCer), iNKT produce large amounts of cytokines involved in antitumor immune responses. Monocyte-derived dendritic cells were loaded with both phosphoantigens (using aminobisphosphonates) and α-GalCer during maturation and subsequently co-cultured with Vγ9Vδ2-T and iNKT cells. Aminobisphosphonates dose-dependently enhanced Vγ9Vδ2-T cell activation, and this was potentiated by α-GalCer-induced iNKT co-activation. iNKT co-activation also enhanced the IFN-γ production and cytolytic potential of Vγ9Vδ2-T cells against tumor cells. Using transwell experiments and neutralizing antibodies cross-talk between iNKT and Vγ9Vδ2-T cells was found to be mediated by TNF-α. Our data provide a rationale for combining both activating ligands to improve Vγ9Vδ2-T cell based approaches in cancer-immunotherapy.
Introduction

Vγ9Vδ2-T cells account for 1-5% of peripheral blood mononuclear cells (PBMC) of healthy adults and are the predominant γδ-T cell subset in human peripheral blood. Vγ9Vδ2-T cells can be activated and expanded by natural (isopentenyl pyrophosphate (IPP)) and synthetic (e.g. BrHPP/Bromohydrin pyrophosphate and HMBPP/Hydroxy-methyl-butenyl-pyrophosphate) phosphoantigens (phosphoAg). Notably, phosphoAg are over-expressed by many human tumor cells, possibly reflecting a state of raised metabolic stress, and can provoke the secretion of cytotoxic molecules by Vγ9Vδ2-T cells. Other compounds, such as aminobisphosphonates (ABP), sensitize target cells to Vγ9Vδ2-T cell killing by promoting the intracellular accumulation of endogenous phosphoAg by inhibiting mevalonate metabolism. Upon stimulation, Vγ9Vδ2-T cells acquire the capacity to kill solid tumors of diverse origins such as squamous cell carcinoma of the head and neck (HNSCC), melanoma, renal cell-, colon-, and breast carcinoma, suggesting Vγ9Vδ2-T cells to be important antitumor effector cells. The synthetic phosphoAg BrHPP was evaluated in phase 1-2 clinical trials in advanced cancer. Intravenous administration of BrHPP activates and expands Vγ9Vδ2-T cells in patients that are simultaneously treated with low-dose s.c. IL-2, and can occasionally induce clinical responses. However, up to now Vγ9Vδ2-T cells show limited consistent efficacy in cancer therapy. Still, their abundance and high in vitro antitumor potential warrant further studies into ways to boost their antitumor effects.

Invariant natural killer T (iNKT) cells are characterized by the expression of a highly restricted T cell antigen receptor (TCR) repertoire in combination with the NK cell marker CD161. This restricted TCR repertoire consists of Vα14.Jα18 paired with Vβ2, Vβ7 or Vβ8.2 in mice and Vα24.Jα18 preferentially paired with Vβ11 in man. Both human and mouse iNKT cells recognize certain glycolipid antigens (Ag), which include the synthetic α-galactosylceramide (α-GalCer), in the context of the monomorphic non-classic MHC class I-like Ag presenting molecule CD1d. iNKT cells play crucial roles in various immune responses, including antitumor immune responses. Their diverse regulatory role in immune responses (either stimulatory or inhibitory) has been attributed to an apparent flexibility of iNKT cells with regards to their predominant cytokine profile. Activation of iNKT cells via α-GalCer in the context of the CD1d Ag presenting molecule results in a cytokine storm in which iNKT cells rapidly produce large amounts of cytokines (e.g. IFN-γ), resulting in the activation of NK cells, CD4+ and CD8+ T cells, B cells, neutrophils, macrophages and the DC with which they interact (reviewed).

Here, we evaluated whether activation of iNKT cells can be used in order to enhance the effector cell function of Vγ9Vδ2-T cells. It is our hypothesis that by targeting both cell types, we will be able to design a treatment strategy which generates more potent antitumor immune responses.
Material and Methods

Generation of moDC
Immature monocyte derived (mo) DC were generated by allowing PBMC to adhere to culture flasks for 2 h at 37 °C. Adherent cells were cultured for 5-7 days in the presence of recombinant human (rh)IL-4 (10 ng/ml, R&D systems, Minneapolis, USA) and rhGM-CSF (100 ng/ml, Bayer AG, Leverkusen, Germany) in IMDM (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Amsterdam, The Netherlands), 100 I.E./ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100 μg/ml streptomycin sulphate (Radiumfarma-Fisiopharma, Naples, Italy), 2.0 mM l-glutamine (Invitrogen, Breda, The Netherlands) and 0.01 mM 2-mercapoethanol (Merck, Darmstadt, Germany), hereafter referred to as complete medium. Immature moDC were matured with 100 ng/ml Lipopolysaccharide (LPS, SIGMA, St. Louis, Missouri, USA) during 24-48 h at 37°C in a humidified atmosphere under 5 % CO₂. Mature moDC were harvested by 5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS, Braun Melsungen AG, Melsungen, Germany), irradiated (5000 Rad), washed in PBS and used for flow-cytometric analysis or for co-culturing experiments.

Generation of Vγ9Vδ2-T cell lines
Vγ9Vδ2-T cell lines were generated from human PBMC by magnetic activated cell sorting (MACS) using either the murine anti-human Vδ2 TCR or anti-human Vγ9 TCR monoclonal antibody (mAb) (Beckman Coulter, Brea, California, USA), combined with a polyclonal Goat-anti-Mouse Ab or anti-phycoerythrin (PE) Ab labelled with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). For culture/expansion of Vγ9Vδ2-T cells pamidronate (PCH, Pharmachemie BV, Haarlem, The Netherlands) was added to immature moDC during maturation with LPS and co-cultured with Vγ9Vδ2-T cells with rhIL-2 (50-100 U/ml, BioVision, Mountain View, California, USA). Purified cells were cultivated by weekly restimulation with Ag loaded mature moDC in a 1:5 ratio. Purity of Vγ9Vδ2-T cells used for experiments was >90%.

Generation of iNKT cell lines
For iNKT cell (defined as Vα24·Vβ11+) expansion experiments PBMC were MACS-sorted using the 6B11 mAb (kind gift of Mark Exley, BIDMC, Harvard Medical School, Boston), or the murine anti-human TCR Vα24-chain mAb (Beckman Coulter), combined with a polyclonal Goat-anti-Mouse Ab labelled with magnetic beads (Miltenyi Biotec). For culture/ expansion of iNKT cells α-GalCer (100 ng/ml, Funakoshi Co, Tokyo, Japan) was added to immature moDC during maturation and co-cultured with iNKT cells with rhIL-2 (50-100 U/ml). Purified cells were cultivated by weekly restimulation with Ag loaded mature moDC in a 1:5 ratio. Purity of iNKT cells used for experiments was >90%.
Activated iNKT cells promote Vγ9Vδ2-T cells through TNF-α

**Antibodies, flow-cytometry and intracellular IFN-γ/ TNF-α/ IL-4/ Granzyme B (GrB) assay**

PE- or fluorescein isothiocyanate (FITC)-labeled Abs directed against murine IgG1, CD3, CD14, CD1a, CD25 (BD Biosciences, New Jersey, USA), Vα24, Vβ11, Vδ2, (Beckman Coulter) and Vγ9 (Biolegend, San Diego, USA) were used for flow cytometry analysis. Ab staining was performed in PBS supplemented with 0.1% BSA and 0.02% sodium-azide for 30 min at 4°C. In order to perform intracellular stainings, Vγ9Vδ2-T cells were placed in a 96-well round-bottom plate in the presence of 0.5 μl of Golgi Plug (BD Biosciences). After 5 h, cells were harvested, washed and stained with surface membrane mAb. After fixation with Cytofix/Cytoperm solution and permeabilization with Perm/Wash solution (both from BD Biosciences), cells were stained with PE-conjugated mAbs specific for IFN-γ, IL-4, or TNF-α (BD Biosciences). To determine Granzyme B expression, permeabilized cells were stained with PE-conjugated anti-human Granzyme B (Pelicluster, Sanquin, Amsterdam, The Netherlands) or the appropriate isotype control. All stained cells were analyzed on a FACS Calibur (BD Biosciences) using CellQuest software.

**Vγ9Vδ2-T and iNKT cell co-culture experiments**

Immature moDC were matured with 100 ng/ml LPS in the presence of either α-GalCer (100 ng/ml), an equal amount of vehicle control, and/or 50-100 μM ABP (added during the final 8-10 h of maturation) as indicated. 5 x 10⁴ Mature Ag-loaded moDC were co-cultured in a 24 wells plate with resting (defined as ≥ 6 days post re-stimulation) iNKT and Vγ9Vδ2-T cell lines (ratio 1:2) for 24 h and subsequently harvested for further analyses. For transwell assays 5 x 10⁴ mature α-GalCer or vehicle loaded moDC were cultured in a 24 wells plate for 24 h with resting iNKT cells (ratio 1:2) in the lower part of a transwell (0.4 μm pore size, Costar, Corning Incorporated), while Vγ9Vδ2-T cells (ratio 1:2) were cultured in the upper part of the transwell. For analyses of the effect of various neutralizing Abs, optimal concentrations in a range of 10-100 μg/ml of anti-human IL-12p70, IL-10, IFN-γ (R&D) and/or anti-human TNF-α (Infliximab, Remicade®, Centocor, Leiden, The Netherlands), were added as indicated. Activation was determined by CD25 expression on Vγ9Vδ2-T cells. Figures show fold increase in percent positive from baseline level.

**CD107a degranulation assay**

Cytolytic degranulation of Vγ9Vδ2-T cells was determined by measurement of the cumulative cell surface exposure of granular membrane protein CD107a/lysosomal-associated membrane protein-1 (LAMP-1). Vγ9Vδ2-T cells were cultured, separated from the combination of iNKT cells and α-GalCer or vehicle loaded mature moDC, in a transwell in the presence or absence of neutralizing anti-human TNF-α mAb. The adherent and Vγ9Vδ2-T cell susceptible renal cell carcinoma cell line, 786-0 (authenticated by the ATCC), was cultured in complete medium. After a 24 h transwell co-culture of iNKT and Vγ9Vδ2-T cells, 4 x 10⁴ plated 786-0 tumor cells and isolated Vγ9Vδ2-T (ratio 1:1) were co-cultured for 5 h at 37°C in the presence of anti-
CD107a-PE (BD Biosciences) and 0.5 μl of Golgi Stop. Subsequently, cells were washed and stained with allophycocyanin (APC)-labeled anti-CD3 mAb and analyzed by flow-cytometry.

**Statistical analysis**
All data were analyzed using paired or unpaired Student T-tests, as appropriate; $P<0.05$ was considered statistically significant.

**Results**

**Activated iNKT cells enhance $V\gamma9V\delta2$-T cell activation.**
Resting (≥ 6 days post re-stimulation) iNKT and $V\gamma9V\delta2$-T cells were co-cultured in the presence of α-GalCer or vehicle loaded mature moDC for 24 h. As shown in Figure 1A activation of iNKT cells by α-GalCer loaded moDC resulted in the simultaneous activation of $V\gamma9V\delta2$-T cells. Of note, when $V\gamma9V\delta2$-T cells were activated by phosphoAg expressing moDC the expected $V\gamma9V\delta2$-T cell activation could be substantially potentiated by co-activation of iNKT cells ($p = 0.00001$; $n = 2$ for 5 and 20 μM ABP and $n = 4$ for 0 and 100 μM ABP) (Figure 1A).

**Potentiation of $V\gamma9V\delta2$-T cell activation by activated iNKT cells does not require cell-cell contact.**
In order to assess whether the co-activation of $V\gamma9V\delta2$-T cells was cell-cell contact dependent or mediated by (a) soluble factor(s), transwell assays were performed. In a transwell, mature α-GalCer or vehicle loaded moDC were co-cultured with resting iNKT cells in the lower part, separated from resting $V\gamma9V\delta2$-T cells in the upper part. Figure 1B shows that α-GalCer induced iNKT cell activation resulted in a similar level of $V\gamma9V\delta2$-T cell activation irrespective of whether or not $V\gamma9V\delta2$-T cells were in direct contact with moDC/iNKT cells ($p = 0.26$), demonstrating that the increased $V\gamma9V\delta2$-T cell activation induced by activated iNKT cells is mediated by (a) soluble factor(s). Figure 1C shows representative dot-plots illustrating the purity of the employed $V\gamma9V\delta2$-T and iNKT cell lines as well as a representative example of the observed changes in CD25 expression on $V\gamma9V\delta2$-T cells.

**Potentiation of $V\gamma9V\delta2$-T cell activation results from TNF-α production by iNKT cells.**
Next, we determined the soluble factor(s) responsible for mediating this stimulatory effect of activated iNKT cells on $V\gamma9V\delta2$-T cells. For this purpose, the inhibitory effect of neutralizing antibodies against several cytokines was assessed. These were selected based on their reported role in crosstalk of iNKT cells with other cell populations and/or their known effects on $V\gamma9V\delta2$-T cells. Figure 2A shows that neutralization of IL-10, IL-12p70, IFN-γ or combinations thereof, did not affect the stimulatory effect of iNKT cells on
Activated iNKT cells promote Vγ9Vδ2-T cells through TNF-α and neutralization of TNF-α resulted in a complete inhibition of the iNKT cell mediated increase in Vγ9Vδ2-T cell activation (Figure 2A and B).

**Figure 1. Activated iNKT cells enhance Vγ9Vδ2-T cell activation.** A) Mature moDC loaded with α-GalCer/phosphoAg/vehicle control were co-cultured with resting iNKT and Vγ9Vδ2-T cell lines. Vγ9Vδ2-T cell CD25 expression was then assessed by flow-cytometry. White bars indicate vehicle loaded moDC; black bars indicate α-GalCer loaded moDC. Shown are means ± SEM (p = 0.00001; n = 2 for 5 and 20 μM ABP and n = 4 for 0 and 100 μM ABP). B) Mature α-GalCer (■) or vehicle (□) loaded moDC were co-cultured with resting iNKT and Vγ9Vδ2-T cells, or were cultured with resting iNKT cells in the bottom of a transwell, separated from Vγ9Vδ2-T cells in the upper part of a transwell. After 24 h Vγ9Vδ2-T cell CD25 expression was assessed by flow-cytometry. Shown are means ± SEM (vehicle v α-GalCer in transwell p = 0.047; n = 5, vehicle v α-GalCer in co-culture p = 0.0002; n = 8; α-GalCer transwell v co-culture p = 0.26). C) Representative dot-plots illustrating the purity of the employed Vγ9Vδ2-T and iNKT cell lines assessed after lymphocyte gating based on forward- and side-scatter properties (upper panel). The lower panels depict representative dot-plots of Vγ9Vδ2-T cell CD25, IFN-γ and CD107a expression after a 24 h co-culture with iNKT cells in the presence (right) or absence (left) of 100 ng/ml α-GalCer. Representative dot-plots of changes in CD25, IFN-γ, and CD107a expression are from 4, 6, and 8 experiments respectively.
Although it is known that iNKT cells can produce high levels of TNF-\(\alpha\) upon triggering \([12]\), we used intracellular cytokine stainings to confirm that iNKT cells and not moDC were responsible for the production of TNF-\(\alpha\) upon activation in this culture system \((p = 0.02; n = 3)\) (Figure 3 A). Furthermore, although it is well known that TNF-\(\alpha\) can directly stimulate \(\gamma\delta\) T cells\(^{16}\) we confirmed that blocking TNF-\(\alpha\) receptor type 2 (using anti-TNFR2 mAb, R&D systems), which is the most abundant of the two types of TNF-\(\alpha\) receptors on \(\gamma\delta\) T cells, before adding TNF-\(\alpha\), indeed inhibited \(\gamma\delta\) T cell activation \((p = 0.03; n = 7, \text{ data not shown})\).
Activated iNKT cells promote Vγ9Vδ2-T cells through TNF-α

Crosstalk between iNKT and Vγ9Vδ2-T cells enhances IFN-γ production and cytotoxicity of Vγ9Vδ2-T cells.

A separate set of experiments was performed to determine functional changes in Vγ9Vδ2-T cells activated by iNKT cells. For this purpose, resting Vγ9Vδ2-T cells were co-cultured in a transwell with resting iNKT cells and α-GalCer or vehicle loaded mature moDC as indicated in subsection 2.5. Activation of iNKT cells by α-GalCer loaded moDC resulted in a significant increase \((p = 0.008; n = 4)\) in IFN-γ production by Vγ9Vδ2-T cells which could be significantly inhibited by neutralizing TNF-α \((p = 0.03; n = 4)\) (Figure 4A). IL-4 production by Vγ9Vδ2-T cells was not affected by co-activation of iNKT cells \((p = 0.12; n = 5)\) (data not shown). The cytolytic potential of Vγ9Vδ2-T cells was evaluated by harvesting the Vγ9Vδ2-T cells, activated as described in subsection 2.6, for a subsequent 5 h co-
culture with Vγ9Vδ2-T cell susceptible 786-0 tumor target cells (renal cell carcinoma cell line). The amount of degranulation was then assessed by measuring Vγ9Vδ2-T cell CD107a expression. Figure 4B shows that the activation of iNKT cells by α-GalCer loaded moDC resulted in a significant increase in Vγ9Vδ2-T cell CD107a expression ($p = 0.006; n = 7$). Again, this effect could be inhibited by neutralization of TNF-α ($p = 0.004; n = 7$). Vγ9Vδ2-T cells which were cultured for 5 h in complete medium, in the absence of 786-0 tumor cells, showed a minimal amount of degranulation, demonstrating specific degranulation upon target cell recognition. A 7-AAD based cytotoxicity assay confirmed data obtained using the CD107a degranulation assay (percent lysis: vehicle 38.4 ± 5.5; α-GalCer 49.5 ± 5.4; α-GalCer + anti-TNF-α 43.3 ± 3.4; vehicle α-GalCer $p = 0.02$; vehicle α-GalCer plus anti-TNF-α $p = 0.37; n = 4$). Representative dot-plots illustrating the observed changes in Vγ9Vδ2-T cell IFN-γ and CD107a expression are shown in Figure 1C (lower panel). Granzyme B expression by resting Vγ9Vδ2-T cells was already >90% at baseline, and no significant changes were observed after co-activation by iNKT cells ($p = 0.12; n = 5$) (data not shown).

**Discussion**

Vγ9Vδ2-T cells constitute the predominant γδ-T cell subset in human peripheral blood. Vγ9Vδ2-T cells can be activated by phosphoAg and subsequently mediate immunoregulatory and antitumor effector functions.17,18 The results from phase 1-2 clinical trials concerning the use of the phosphoAg BrHPP in advanced cancer show that i.v. administration of BrHPP activates and expands Vγ9Vδ2-T cells in patients who are simultaneously treated with low-dose s.c. IL-2.1 Though some clinical responses were noted in these trials, overall, this Vγ9Vδ2-T cell based approach still showed limited efficacy in cancer therapy. It is therefore critical to determine ways to potentiate the antitumor effector functions of Vγ9Vδ2-T cells.

Here, we analyzed whether iNKT cells, an important pro-inflammatory regulatory T cell subset, can potentiate the antitumor effector functions of Vγ9Vδ2-T cells. Activated iNKT cells are known to influence the function of conventional T, DC and NK cells, facilitating the initiation and propagation of antitumor immune responses. Activated iNKT cells upregulate the expression of CD40L and cytotoxic molecules. As a consequence, CD40L on iNKT cells activates DC via CD40, which in turn promotes IFN-γ production by iNKT cells and subsequently stimulates NK cells and CD8+ cytotoxic T lymphocytes (CTL), mediating antitumor cytotoxicity.19-21 Crosstalk between iNKT and B cells is predominantly mediated by IL-422 Depending on the employed model, several mechanisms have been reported to be responsible for crosstalk between iNKT and regulatory T cells (Tregs), including IL-4, IL-10, IL-2, IFN-γ, and the CXCL10 pathway.23-25 The interaction between iNKT cells and Tregs has been thoroughly investigated in the context of graft-versus-host-disease (GVHD) in leukaemia patients; host iNKT cells induce an IL-4-dependent expansion of donor Tregs that protects against GVHD.26
Our data show that, in addition to the effect on conventional T cells, NK cells, B cells, DC and Tregs, iNKT cells can lead to an enhancement of the anti-tumor effector function of Vγ9Vδ2-T cells. In contrast to the crosstalk between iNKT cells and the above mentioned cells, crosstalk between iNKT and Vγ9Vδ2-T cells is mediated by TNF-α. Though iNKT cell-derived TNF-α has been implicated in the regulation of osteoclast function27 and liver injury in mice28, we here, for the first time, report its direct relationship with Vγ9Vδ2-T cell activation.

Figure 4. Crosstalk between iNKT and Vγ9Vδ2-T cells enhances IFN-γ production and degranulation of Vγ9Vδ2-T cells. A) Mature α-GalCer or vehicle loaded moDC were cultured for 24 h with resting iNKT and Vγ9Vδ2-T cells in the presence or absence of neutralizing anti-human TNF-α mAb. Intracellular IFN-γ expression was assessed in Vγ9Vδ2-T cells by flow-cytometry. Shown are means + SEM (vehicle v α-GalCer p = 0.008; α-GalCer v a-GalCer plus neutralizing anti-human TNF-α mAb p = 0.03; n = 4). B) Mature α-GalCer or vehicle loaded moDC were cultured for 24 h with resting iNKT cells in the bottom of a transwell in the presence or absence of neutralizing anti-human TNF-α mAb, separated from Vγ9Vδ2-T cells in the upper part of a transwell. After 24 h Vγ9Vδ2-T cells were harvested and added to adhered 786-0 tumor cells. After 5 h incubation the amount of degranulation (CD107a) of Vγ9Vδ2-T cells was assessed by flow-cytometry. Shown are means + SEM (vehicle v α-GalCer p = 0.006; α-GalCer v α-GalCer plus neutralizing anti-human TNF-α mAb p = 0.004; n = 7). □ indicates Vγ9Vδ2-T cells incubated with complete medium only (n = 6).
One can envision that this stimulatory effect of iNKT cells on the effector function of \(V\gamma9V\delta2\)-T cells can be used to strengthen future \(V\gamma9V\delta2\)-T cell based immunotherapeutic approaches, e.g. by co-loading moDC with \(\alpha\)-GalCer and phosphoAg. The combined presence of activated iNKT and \(V\gamma9V\delta2\)-T cells at the effector site could potentiate anti-tumor immune responses. This strategy is broadly applicable, since both iNKT and \(V\gamma9V\delta2\)-T cells can kill a wide variety of tumor targets and have well conserved Ag recognition receptors. Another advantage of this strategy, besides the enhancement of the anti-tumor effector function of \(V\gamma9V\delta2\)-T cells, is the ability of \(V\gamma9V\delta2\)-T cells to act as professional APC at the tumor site. The APC function in human \(V\gamma9V\delta2\)-T cells has been described by Brandes et al. and might be highly relevant to immunotherapy.29

In conclusion, our data provide a rationale for combining activating ligands for both iNKT and \(V\gamma9V\delta2\)-T cells as a means to strengthen future \(V\gamma9V\delta2\)-T cell based approaches in cancer immunotherapy.

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